PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : A61K 37/02, 39/21, C07K 13/00, 15/04 — A1

(11) International Publication Number: WO 94/04121

(43) International Publication Date: 3 March 1994 (03.03.94)

(21) International Application Number: PCT/US93/07786

(22) International Filing Date: 17 August 1993 (17.08.93)

(30) Priority data:
931,217 17 August 1992 (17.08.92) US

(71) Applicant: AUTOIMMUNE, INC. [US/US]; 128 Spring Street, Lexington, MA 02173 (US).

(72) Inventors: HAFLER, David, A. ; 110 Forest Avenue, West Newton, MA 02165 (US). WEINER, Howard, L. ; 114 Somerset Road, Brookline, MA 02165 (US).

(74) Agents: FRANKFORT, Howard, M. et al.; Darby & Darby, 805 Third Avenue, New York, NY 10022 (US).

(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, MG, MN, MW, NL, NO, NZ, PL, RO, RU, SD, SE, SK, UA, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: BYSTANDER SUPPRESSION OF RETROVIRAL-ASSOCIATED NEUROLOGICAL DISEASE

(57) Abstract

The invention is directed to a method of treating a mammal afflicted with a retroviral-associated neurological disease through the administration of an effective amount of a bystander antigen. The bystander antigen is selected such that it elicits the release of transforming growth factor beta (TGF-β) from suppressor T-cells, and, preferably, targets the T-cells to the loci in the body (i.e., the neural tissue) of the mammal which is under cytotoxic attack during the neurological disease. The released TGF-β suppresses the cells responsible for the cytotoxic attack, thus reducing the neurological disease.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NE	Niger
BE	Belgium	GN	Guinea	NL	Netherlands
BF	Burkina Faso	GR	Greece	NO	Norway
BG	Bulgaria	HU	Hungary	NZ	New Zealand
BJ	Benin	IE	Ireland	PL	Poland
BR	Brazil	IT	Italy	PT	Portugal
BY	Belarus	JP	Japan	RO	Romania
CA	Canada	KP	Democratic People's Republic of Korea	RU	Russian Federation
CF	Central African Republic			SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovak Republic
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	UA	Ukraine
DE	Germany	MG	Madagascar	US	United States of America
DK	Denmark	ML	Mali	UZ	Uzbekistan
ES	Spain	MN	Mongolia	VN	Viet Nam
FI	Finland				

BYSTANDER SUPPRESSION OF RETROVIRAL-ASSOCIATED NEUROLOGICAL DISEASE

This application is a continuation-in-part of Weiner et al., U.S. Patent Application Serial No. 843,752, filed February 28, 1992; Weiner et al., U.S. Patent Application Serial No. 487,732, filed March 2, 1990 pending; and Weiner et al., U.S. Patent Application Serial No. 454,806 filed December 20, 1989, pending.

Field of the Invention

This invention pertains to an improvement in the treatment of retroviral-associated neurological disease. More specifically, the invention is directed to the use of bystander antigens (i.e. antigens that suppress cells involved in the destruction of neurological tissue) in the treatment of neural damage associated with infection with retroviruses.

Background of the Invention

Retroviral infections are often associated with neurological manifestations which stem from lesions in nervous tissue. It is known that infection with retroviral agents can serve to systemically activate large numbers of T-cells. It is theorized that the tissue destruction caused by neurotropic retroviral infections is due to a non-specific cytotoxic attack mediated by endogenous lymphokines and cytokines released from these activated T-cells. Two retroviruses known to elicit such

neural damage are human leukemia virus type I (HTLV-I) and human immunodeficiency virus (HIV).

Infection with human leukemia virus type I (HTLV-I) is associated with a neurological disorder, termed HTLV-I-associated myelopathy (HAM). This condition is thought to be identical to a previously identified neurological disease--tropical spastic paraparesis (TSP). These conditions are characterized by weakness and spasticity of the extremities, exaggerated reflexes, urinary/fecal incontinence, mild peripheral sensory loss, as well as other neurological defects. Anti-HTLV-I antibodies and HTLV-I antigens have been detected in the cerebrospinal fluid (CSF) of these patients. Southern hybridization and polymerase chain reaction (PCR) analysis has confirmed the presence of HTLV-I in the blood and lymphocytes of the CSF. The neurological symptoms appear to stem from lesions in the brain and spinal cord of infected individuals. (Cann et al., "Human T-Cell Leukemia Virus Types I and II", chapter 52 of Virology, Fields et al. (eds), second edition, Raven Press Ltd., New York, 1990) It is thought that these lesions are possibly caused by a cytotoxic, T-cell mediated immune reaction against HTLV-I-infected cells in the central nervous system. (Bhagaviati et al., New Eng. J. Med. 318(18): 1141-1147, 1988)

Some clinical benefit has been seen in patients suffering from these conditions with treatment with oral corticosteroids, drugs which non-specifically suppress immune responses. However, only limited improvement is seen and such drugs have significant toxicity. (Cann et al., supra) This toxicity results in serious side effects including the possibility of inducing global immunosuppression of the patient. In other words, prolonged treatment with such drugs downregulates the normal protective immune response against pathogens thereby increasing the risk of infections. In addition, patients subjected to prolonged global immunosuppression have an increased risk of developing severe medical complications from

the treatment, such as malignancies, kidney failure, and diabetes.

Infection with HIV, the viral agent implicated in Acquired Immunodeficiency Syndrome (AIDS), is also associated with neurological disorders affecting both the peripheral nervous system (i.e., chronic distal symmetric polyneuropathy, chronic inflammatory myelinating polyneuropathy) and the central nervous system (i. e., vacuolar myelopathy). Clinical features depend on the tissue comprising the lesions, but can involve cognitive deficits and memory loss, dementia, psychomotor slowing, weakness, sensory deficits, spasticity of the extremities, exaggerated reflexes, urinary/fecal incontinence, as well as other neurological defects. (Hirsch et al., "HIVs: Biology and Medical Aspects", chapter 54 of Virology, Fields et al. (eds), second edition, Raven Press Ltd., New York, 1990) The pathogenesis of HIV-associated neurological disease is not entirely understood. However, there is an increase in the levels of cytokines in the CNS with infection by HIV. This increase appears to be indicative of a relative state of "immune activation" in the brain of HIV-positive individuals as compared to HIV-negative individuals, as documented by immunocytochemical analysis of brain tissue. (Tyor et al., Annals of Neurology 31:349-360, 1992) A hypothesized source of the increased cytokine levels is secretion by HIV-infected macrophages which have migrated into the CNS.

There is no effective treatment for HIV infection. Treatment with the antiviral agent 3' azido-3'-deoxythymidine, also known as AZT, has been shown to decrease opportunistic infections and may be influential in prolonging the time the patient is symptom-free. With respect to neurological symptoms, AZT has been reported to lead to some improvement of HIV dementia complex, but this effect was not seen with all types of HIV-associated neurological disease (Yarchoan et al., Lancet 1:132-135, 1987). Additionally, HIV is highly mutagenic, so it is uncertain how long this treatment will

remain effective. Further, the treatment is not without side effects, as AZT is a toxic agent, often causing reduction in both red and white blood cell counts. (Hirsch et al., supra)

Treatment with other nucleoside analogs, specifically dideoxycytidine (ddC) and dideoxyinosine (ddI), have been shown to have positive effects on neurological manifestations of HIV infection. (Yarchoan et al., J. Cell Biochem. suppl. 0 (14 Part D), p. 93, 1990) Again, resistent strains of HIV develop and the treatment results in side effects due to toxicity. Therefore, there remains a need for an effective, non-toxic method of treatment for HIV-associated neurological disease.

As disclosed in co-pending U.S. Patent Application Serial No. 07/843,752, filed February 28, 1991, the present inventors and their coworkers have devised methods and pharmaceutical formulations useful for treating autoimmune diseases based on the concept of oral tolerization (or tolerization by inhalation). This method utilizes bystander antigens (defined below) as tolerizers, either alone or in combination with so-called "synergists", i.e., compounds which enhance the tolerizing effect of the bystander antigens. It has been discovered that the bystander antigens do not need to be autoantigens, i.e., they do not need to be the an antigen that is under attack by the disease-inducing cells. It is an interesting feature of the oral tolerization method that oral administration of a bystander antigen can stave off tissue damage done by cells specific for another antigen or antigen fragment. It is this aspect of the oral tolerization method which allows application of this method to diseases which are not considered to be autoimmune, such as retroviral-associated neurological disease. As will be discussed in detail below, it is believed that the suppression of T-cell activity with the administration of bystander antigen alleviates non-specific cytotoxic attacks, such as those observed in nervous tissue in association with retroviral infection. By reducing the cytotoxic attacks on the nervous tissue, there is a reduction

of lesion formation, thus reducing the neurological disease which is thought to result from such damage to nervous tissue. The cellular mechanism for this suppression is discussed below and illustrated in Examples 1 and 2. The application of this method to treatment and prevention of retroviral-associated neurological disease is also discussed below, together with experimental data to support the role of activated T-cells in the pathology of these diseases.

Therefore, it is an object of the present invention to provide improved methods used to treat or alleviate the symptoms of individuals suffering from retroviral-associated neurological disease. Said methods involve the use of compositions comprising bystander antigens which are used alone or optionally in combination with synergists and other immune response regulators.

Summary of the Invention

The present invention is based on the unexpected and surprising discovery that oral or enteral administration (or administration by inhalation) of certain antigens (called "bystander antigens") causes suppressor T-cells to be elicited that in turn suppress the T-cells that contribute to non-specific cytotoxic attack of a tissue, such as that which can occur with neurotropic retroviral infection. The T-cells elicited by the bystander antigen mediate the release of transforming growth factor beta (TGF-β). This factor non-specifically suppresses the activity of all immune cells in the area, including the T-cells responsible for cytotoxic attack of tissue. By targeting the elicited suppressor T-cells to neural tissue (i.e., the brain, spinal cord, peripheral nerves or associated cell types), cytotoxic damage to these tissues can be reduced. The method of targeting the T-cells is discussed below. It is in this way that oral administration of bystander

antigens can be used as treatment for retroviral-associated neurological disease.

For this type of suppression mechanism to work, it is not necessary that the TGF-β releasing T-cells recognize the same antigen as the disease-contributing T-cells in neural tissue . All that is necessary is that both types of cells be found in the same vicinity when TGF-β is released. One way to achieve this is to use as the bystander antigen an antigen that (a) has the ability to elicit T-cells that cause release of TGF-β and (b) is itself specific to the neural tissue under attack, that is, an antigen presented by class I or class II MHC to be recognized by $CD8^+$ or $CD4^+$ T-cells, respectively. It is in this way that the suppressor T-cells that are elicited pursuant to oral administration of the bystander antigen (and that release the T-cell suppressing TGF-β) will be directed to neural tissue. By targeting the TGF-β to neural tissue, the T-cells located in this area and responsible for damage to this tissue will be suppressed, thus reducing cytotoxic tissue damage.

Therefore, one aspect of the present invention is directed to a method for treating retroviral-associated neurological disease in a mammal, the method comprising administering to said mammal an effective amount for treating said disease of a bystander antigen, said antigen eliciting the release of TGF-β from T-cells at a locus within the body of said mammal wherein said TGF-β suppresses the T-cells contributing to said cytotoxic response.

In another aspect, the present invention provides a method for administering an effective amount for treating retroviral-associated neurological disease of a bystander antigen in a pharmaceutical oral dosage form, said antigen upon administration eliciting the release of TGF-β from T-cells at a locus within the body of said mammal wherein said TGF-β suppresses the T-cells contributing to said cytotoxic response.

In yet another aspect, the present invention provides a method for administering an effective amount for treating retroviral-associated neurological disease of a bystander antigen in a pharmaceutical inhalable dosage form, said antigen upon administration eliciting the release of TGF-β from T-cells at a locus within the body of said mammal wherein said TGF-β suppresses the T-cells contributing to said cytotoxic response.

Brief Description of the Drawings

Figure 1 is a bar graph showing the in vitro suppression of proliferative responses mediated by supernatants of lymphocytes or lymphocyte subsets isolated from orally tolerized animals.

Figure 2 is a bar graph showing the inhibition of in vitro suppression by anti-Transforming Growth Factor-beta (TGF-β) antibody.

Figure 3 is a bar graph showing TGF-β activity in serum-free culture supernatants of suppressor T-cells isolated from orally tolerized animals.

Figure 4 (A-D) depicts a series of graphs showing the effects of anti-TGF-β antibodies and control sera on experimental allergic encephalomyelitis (EAE) in orally tolerized (MBP-fed) and non-MBP-fed animals.

Figure 5 (A-D) depicts a series of bar graphs showing the effect of anti-TGF-β antibodies on Delayed Type Hypersensitivity (DTH) responses in orally tolerized and control animals.

Figure 6 (A-C) depicts a series of graphs showing suppression of autoimmune disease associated with oral administration of a bystander antigen and substantially simultaneous immunization with MBP followed by injection of selected antigens.

Figure 7 is a bar graph showing Delayed Type Hypersensitivity (DTH) responses associated with such bystander suppression.

Figure 8 is a bar graph showing whether in vivo bystander suppression of EAE is associated with bovine serum albumin (BSA), ovalbumin (OVA) and myelin basic protein (MBP) fed animals immunized with MBP and injected with the same antigen as was fed.

Figure 9 is a bar graph showing that the adoptive transfer of bystander suppression is associated with $CD8^+$ suppressor T-cells.

DETAILED DESCRIPTION OF THE INVENTION

All patent applications, patents, and literature references cited in this specification are hereby incorporated by reference in their entirety. In case of inconsistencies, the description including the definitions and interpretations of the present disclosure will prevail.

Definitions

The following terms used in this disclosure shall have the meaning ascribed to them below:

(a) "Bystander antigen" or "bystander" is a protein, protein fragment, peptide, glycoprotein, or any other immunogenic substance (i.e. a substance capable of eliciting an immune response) that (i) upon oral or enteral administration (or administration by inhalation) elicits suppressor T-cells that cause TGF-β to be released and thereby suppress cells that contribute to destruction of tissue. Preferably, the suppressor T-cells elicited by the bystander will be targeted to the same tissue that is under attack during the disease.

(b) "Bystander suppression" is suppression of cells that contribute to tissue destruction by the release of the immunosuppressive factor TGF-β, this release being in turn mediated by suppressor T-cells elicited by the ingestion or inhalation of a bystander antigen and recruited to the site where cells contributing to the tissue destruction are found.

The result is downregulation of the non-specific cytotoxic response.

(c) "Mammal" is defined herein as any organism having an immune system and being susceptible to retroviral infection.

(d) "Treatment" is intended to include both the prophylactic treatment to prevent neurological disease seen with retroviral infection (or to prevent the manifestation of clinical or subclinical, e.g., histological symptoms thereof), as well as the therapeutic suppression or alleviation of symptoms after the onset of such neurological disease.

(e) "Synergists" are defined herein as substances which augment or enhance the suppression of the clinical (and/or histological) manifestation of retroviral neurological disease when administered orally or by inhalation in conjunction with the administration of a bystander antigen. As used in the preceding sentence, and elsewhere in this specification, "in conjunction with" (also referred to herein as in association with) means before, substantially simultaneously with or after oral or aerosol administration of bystander antigens. Naturally, administration of the conjoined substance should not precede nor follow administration of the bystander antigen by so long an interval of time that the relevant effects of the substance administered first have worn off. Therefore, the synergists should be administered within about 24 hours before or after the bystander antigen, and preferably within about one hour.

(f) "Oral" administration includes oral, enteral or intragastric administration.

(g) A disease having the "characteristics" or "symptoms" of retroviral-associated neurological disease refers to a spontaneous or induced disease state that presents with specific inflammation of the same tissue as that afflicted in said neurological disease.

(h) "Retroviral-associated neurological disease" refers to a disease state resulting from lesions present in neural tissue (i.e., the brain, the spinal cord, or peripheral nerves, and associated cells types, such as glial and endothelial cells) which are the result of non-specific cytotoxic attacks mediated by T-cells brought about by the immune response against infection with a neurotropic retrovirus.

(i) "HTLV-I antigen" or "HIV antigen" is a virally derived protein or protein fragment which, upon oral, enteral, or aerosol administration, brings about an appropriate immune response for the treatment method of the present invention, as defined below.

Description of Bystander Suppression

It has now unexpectedly been discovered that the oral or by-inhalation administration of bystander antigens specific for neural tissue is an effective treatment for retroviral-associated neurological disease. Based on these discoveries it is believed that HAM/TSP and HIV-associated neurological disease are excellent candidate diseases for this treatment method. Further, it is believed that such treatment is non-toxic and will not interfere with other therapeutic agents used to treat retroviral-infected individuals such as AZT or pentadiamine, the latter used for the prevention of *Pneumocystis carinii* pneumonia (PCP), an AIDS-associated opportunistic infection.

As is the case for suppression of autoimmune diseases by oral administration of autoantigens and bystander antigens, suppression of retroviral-associated neurological diseases is mediated by oral administration of bystander antigens. This is brought about by elicitation of suppressor T-cells that release an immunosuppressive factor, transforming growth factor-beta (TGF-β). TGF-β is not specific for the antigen triggering the suppressor cells that release it, even though these suppressor T-cells release TGF-β only when triggered by the orally

administered (or inhaled) antigen. Recruitment of the suppressor T-cells to a locus within a mammal where cells contributing to the cytotoxic destruction of tissue are concentrated allows for the release of TGF-β to be in the vicinity of the disease-causing cells and suppresses (i.e. shuts down) these cells. The ability of TGF-β to suppress these "destructive" cells is independent of the antigen for which the destructive cells may be specific.

The preferred way to accomplish suppression of the destructive cells is to select for oral administration to the mammal an antigen which is not only capable of eliciting suppressor T-cells capable of releasing TGF-β but which is capable of targeting these suppressor T-cells to a location within the mammal's body where destructive cells are found in high concentration. The preferred and most efficient target for the suppressor T-cells is the tissue under cytotoxic attack in the particular retroviral-associated neurological disease involved because the destructive cells will be concentrated in the vicinity of that tissue. Hence, it is preferred that the bystander antigen (to which the suppressor T-cells are specific) be itself an antigen specific to the tissue under attack. For the treatment of HAM/TSP and HIV-associated neurological disease, the preferred bystander antigen for use is myelin basic protein (MBP) or fragments thereof. Specifically preferred for use is the human MBP peptide comprising amino acids 84-99. As MBP is found exclusively in nervous tissue, the elicited T-cells would be targeted to the nervous system, which is the system under cytotoxic attack in these diseases. An alternative preferred bystander antigen for use in the treatment of HAM/TSP and HIV-associated neurological disease is proteolipid protein (PLP) or fragments thereof. PLP is also a component of nervous tissue, and thus would also target the elicited T-cells to the tissue which is under cytotoxic attack. Other potential bystander antigens for use in the present invention are antigenic immunodominant fragments

derived from the HTLV-I or HIV organism. Such fragments are generally small in size, i.e. 8-mer fragments, and can be identified as immunodominant through interaction with the pocket of HLA-A2.1. (Winter et al., J. of Immunology 146:3508-3512, 1991) A preferred antigen of this type for use in treatment of HAM/TSP is peptide 2-25 of the HTLV-1 Tax-1 protein and fragments thereof.

Fragments of MBP, PLP, or of viral peptides for use in the present invention can be identified using the transwell system of Example 1 and 2 below. Fragments which elicit a release of a high amount of TGF-β are those suitable for use in the present invention.

In more detail, the mechanism of bystander suppression according to the present invention for a tissue-specific bystander antigen is as follows: After a tissue-specific bystander antigen is administered orally (or enterally, i.e., directly into the stomach) or by inhalation, it passes into the small intestine, where it comes into contact with the so-called Peyers Patches, which are collections of immunocytes located under the intestinal wall. These cells, are in turn in communication with the immune system, including the spleen and lymph nodes. The result is that suppressor T-cells are induced and recruited to the area of tissue damage, where they cause the release of TGF-β, which can non-specifically downregulate the B-cells as well as the activated T-cells. It is these activated cells that are thought to be responsible for the release of cytotoxic factors which non-specifically destroy the surrounding tissue. By downregulating the activity of these T-cells, the tissue destruction is reduced. Despite the non-specific nature of the activity of TGF-β, the resulting tolerance is specific for the retroviral-associated neurological disease by virtue of the fact that the bystander antigen is specific for the tissue under attack and suppresses the immune cells that are found at or near the tissue being damaged. This mechanism is what is utilized in

the treatment of HAM/TSP and HIV-associated neurological disease, preferably with administration of MBP or fragments thereof, to bring about targeting of the T-cell response to the neural tissue under cytotoxic attack.

TGF-β

TGF-β affects cells of the immune system (e.g., T and B lymphocytes) thereby influencing inflammatory responses. T-lymphocytes (and other cells) produce TGF-β; it is released relatively late in the cascade of immune system response events (after T-cell activation) and is highly suppressive for both T- and B-cell proliferation. Numerous normal tissues have the ability to produce TGF-β. These include human platelets, placenta, bovine kidney, bone, NK cells, B-cells, as well as CD4+ and CD8+ T-cells and activated macrophages. The isolation and biological properties of TGF-β have been described in Transforming Growth Factor-βs Chemistry, Biology, and Therapeutics, Piez, K.A. et al Eds, Ann. N.Y. Acad. Sci. 593:1-217, 1990.

Although TGF-β was initially identified as a growth factor, it soon became clear that it was a substance having many and important immunoregulatory properties including inhibition of B- and T-cells and inhibition of the activity of CD4+ cells more than that of CD8+ cells, both in rodents and humans. TGF-β is also known to antagonize inflammatory cytokines such as tumor necrosis factor (TNF) and gamma interferon (IFN-γ), block cytotoxic lymphocyte activity and inhibit the induction of receptors for Interleukin-1 (IL-1) and Interleukin-2 (IL-2) thereby rendering cells unresponsive to these cytokines. TGF-β is a protein which has a molecular weight of 25 kD and is composed of two identical 12.5 kD subunits that are held together by a number of interchain disulfide bonds. At least two forms of TGF-β exist: active and latent. Active TGF-β has a short half-life and a small volume distribution whereas latent TGF-β has an extended half-life and a larger volume distribution. Two isoforms of TGF-β exist,

TGF-β1 and TGF-β2. It is believed that TGF-β1 is involved in bystander suppression.

Bystander Antigens

Antigens and fragments thereof not specific to the tissue under attack during retroviral-associated neurological disease, yet still useful as bystander antigens, can be identified among nontoxic antigenic substances by using the same assay system as was used for OVA in e.g. Example 1.

Antigens specific for neural tissue for use as bystander antigens in the present invention can be identified by testing the ability of such specific antigens to cause release of TGF-β, which can be detected. For example, one or more potential tissue specific bystander antigens can be purified using well-known antigen purification techniques from the tissue that is the target of cytotoxic attack.

Bystander antigens (as well as fragments and analogs thereof) can also be obtained using recombinant DNA technology, in bacterial, yeast, insect (e.g. baculovirus) and mammalian cells using techniques well-known to those of ordinary skill in the art. Amino acid sequences for many potential and actual bystander antigens are known: e.g., see Appendix A for the sequence of MBP and Tuohy, V.K., et al., J. Immunol. 142:1523-1527, 1989 (encephalitogenic determinant of mouse PLP).

Specifically disclosed in Example 4 is the recombinant production of human MBP for use as bystander antigen in the methods of the present invention. The use of recombinantly-produced human MBP (rMBP) is preferred to the use of protein isolated from brain tissue, as rMBP lacks proteolytic fragments which characterize preparations of the protein isolated from tissue. Further, the use of recombinant technology can easily be adapted for the production of only specific epitopes desired for use as bystander antigens.

The amino acid sequences for bovine PLP; and bovine, human, chimpanzee, rat, mouse, pig, rabbit, and guinea pig MBP,

although taken from published sources, are provided in Appendix A for convenience.

In addition, some tissue-specific antigens are commercially available: e.g. myelin basic protein.

Potential bystander antigens can be tested for suitability. The antigen is fed to mammals, followed by the removal of spleen cells or circulating T-cells from the blood or CSF of these mammals. The cells are stimulated in vitro with the same antigen which was fed to the mammal. T-cells elicited by stimulation can be purified and supernatants can be tested for TGF-β content quantitatively and/or qualitatively using e.g. a suitable commercially available polyclonal or preferably monoclonal antibody raised against TGF-β. Other known assays for TGF-β detection such as that described in Example 1 below, which uses a commercially available mink lung epithelial cell line, can also be used. Such methods for testing for TGF-β production are described in detail in the Examples, below. Those antigens which result in the production of sufficient TGF-β are those suitable for use in the treatment methods of the present invention.

Use of Bystander Antigens - Dosages

The suppression of cytotoxic attack induced by the bystander antigens of this invention is expected to be dose-dependent over a broad range of oral (or enteral) or inhalable dosages. However, it is expected that there will be minimum and maximum effective dosages. In other words, suppression of the clinical and histological symptoms of a retroviral-associated neurological disease will occur within a specific dosage range. However, this range will vary with the retrovirus involved, the mammal being treated, the bystander antigen used for treatment, as well as other factors.

Ascertaining the effective dosage range as well as the optimum amount is well within the skill in the art. For

example, dosages for mammals and human dosages can be determined by beginning with a relatively low dose (e.g., 1 microgram), progressively increasing it (e.g. logarithmically) and measuring the amount of TGF-beta in the blood and/or scoring the disease severity, according to well-known scoring methods (e.g., on a scale of 1 to 5, or by measuring the number of attacks, or by measuring grip strength, stiffness, vision, etc. depending on the type of neurological symptom displayed by the patient). The optimum dosage will be the one generating the maximum amount of TGF-beta in the blood and/or cause the greatest decrease in neurological disease symptoms. An effective dosage range will be one that causes at least a statistically significant attenuation of at least one symptom characteristic of the neurological disease being treated. Such a method can be used to determine the appropriate dosage of bystander antigen for the treatment of HAM/TSP and HIV-associated neurological disease.

The present invention can also be advantageously used to prevent the onset of retroviral-associated neurological disease in susceptible individuals at risk for such complications. Screening methods for infection with HTLV-I and HIV utilizing immunoassays and PCR technology have been developed. Coupled with an association of the development of HAM/TSP with certain HLA haplotypes, individuals with an increased risk of developing the disease with infection can be identified. (Cann et al., supra) Although no such connection with HLA haplotype and neurologic manifestations of HIV infection has been found, neurological complications are more prevalent with HIV infection, occurring in 25%-60% of HIV-infected patients (Hirsch et al., supra). Such a high prevalence may justify general prophylactic treatment against this manifestation of HIV infection. Thus, the present invention has application in the prevention of retroviral-associated neurological disease.

Neural tissue extracts can be used as well as specific bystander antigens. However, administration of one or more individual antigens is preferred.

Thus, according to the present invention, when treating retroviral-associated neurological disease, an effective amount (determined as described above) of an antigen specific for nervous tissue, for example, MBP, can be administered orally or by inhalation.

The present inventors have also discovered that orally administered bystander antigens possess epitopes which specifically induce the production and/or release of TGF-β. Although immunodominant epitopes of e.g., MBP have previously been disclosed, i.e., those epitopes which a majority of patients' CD4+ T lymphocytes recognize and proliferate in response to, or which a majority of a patient's antibodies recognize, immunosuppressive epitopes, i.e., those that elicit the production and/or release of TGF-β, have been identified only in previous applications of the present inventors, and are the preferred source of bystander antigen for the present invention. For human MBP the immunosuppressive epitope encompasses peptides 84-99. Therefore, the oral or inhalation administration of peptides encompassing these epitopes is expected to be more specific in eliciting bystander suppression than administration of the entire antigen. Such immunosuppressive epitopes can be identified in other antigens for use in the instant invention, for example, in HTLV-I or HIV-derived proteins such as tax or gag. It is possible, however, that the specificity imparted by the use of the immunosuppressive epitope would result in a reaction which is too specific and thus detrimental for a particular patient. Therefore, if the sensitivity of a patient to a particular antigen is unknown, whole antigen should be preliminarily used, which could be followed by treatment with an immunosuppressive epitope if desired.

The bystander antigen can be administered in conjunction with synergists which may enhance the effectiveness of the treatment. Non-limiting examples of synergists for use in the present invention include bacterial lipopolysaccharides from a wide variety of gram negative bacteria such as various subtypes of E. coli and Salmonella (LPS, Sigma Chemical Co., St. Louis, MO; Difco, Detroit, MI; BIOMOL Res. Labs., Plymouth, PA), Lipid A (Sigma Chemical Co., St. Louis, MO; ICN Biochemicals, Cleveland, OH; Polysciences, Inc., Warrington, PA) and immunoregulatory lipoproteins, such as peptides covalently linked to tripalmitoyl-S-glycarylcysteinyl-seryl-serine (P_3 C55) which can be obtained as disclosed in Deres, K. et al. (Nature, 342:561-564, 1989) or "Brauns" lipoprotein from E. coli which can be obtained as disclosed in Braun, V., Biochim. Biophys. Acta 435:335-337, 1976. LPS is preferred and Lipid A particularly preferred. Lipid A is particularly preferred for use in the present invention because it is less toxic than the entire LPS molecule. LPS for use in the present invention can be extracted from gram-negative bacteria and purified using the method of Galanes et al. (Eur. J. Biochem. 9:245, 1969) and Skelly, R.R., et al. (Infect. Immun. 23:287, 1979).

The route of administration of the bystander antigens of the present invention is preferably oral or enteral. The preferred oral or enteral pharmaceutical formulations may comprise, for example, a pill or capsule containing an effective amount of one or more of the bystander antigens of the present invention with or without an effective amount of a synergist.

In general, when administered orally or enterally, the bystander antigen may be administered in single dosage form or multiple dosage forms.

The effective amount of a synergist, e.g. LPS or Lipid A, to be administered in conjunction with the bystander broadly ranges between about 0.15 and 15 mg per kg body weight

of said mammal per day and preferably between about 0.3 and 12 mg per kg body weight of said mammal per day.

In an alternative preferred embodiment of the present invention the pharmaceutical formulations or dosage forms of the present invention can also be administered to mammals suffering from retroviral-associated neurological disease by inhalation, preferably in aerosol form. The inhalation mode of administration is preferably not through the nasal passages but through the bronchial and pulmonary mucosa. It is expected that lower amounts of the bystander antigens of the present invention will be required using aerosol administration for treating retroviral-associated neurological disease as this effect has been found when treating experimental autoimmune encephalomyelitis (EAE) with myelin basic protein (MBP) and adjuvant arthritis with collagen as disclosed in co-pending U.S. Patent Application Serial No. 454,486 filed December 20, 1989. The amounts of the bystander antigens of the present invention which may be administered in an aerosol dosage form would be between about 0.1 mg and about 15 mg per kg body weight of a mammal per day and may optionally include a synergist in amounts ranging between about 0.1 and about 15 mg per kg body weight of said mammal per day and may be administered in single dosage form or multiple dosage forms. The exact amount to be administered will vary depending on the state and severity of a patient's disease and the physical condition of the patient.

The route of administration of the bystander antigens according to this alternate embodiment of the present invention is in an aerosol or inhaled form. The bystander antigens and related compounds of the present invention can be administered as dry powder particles or as an atomized aqueous solution suspended in a carrier gas (e.g. air or N_2). Preferred aerosol pharmaceutical formulations may comprise for example, a physiologically-acceptable buffered saline solution containing

between about 1 mg and about 300 mg of the bystander antigens of the present invention.

The methods of the present invention may involve administration of pharmaceutical formulations in the form of an aerosol spray using for example, a nebulizer such as those described in U.S. Patent Nos. 4,624,251 issued November 25, 1986; 3,703,173 issued November 21, 1972; 3,561,444 issued February 9, 1971 and 4,635,627 issued January 13, 1971. The aerosol material is inhaled by the subject to be treated.

Other systems of aerosol delivery, such as the pressurized metered dose inhaler (MDI) and the dry powder inhaler as disclosed in Newman, S.P. in Aerosols and the Lung, Clarke, S.W. and Davia, D. eds. pp. 197-224, Butterworths, London, England, 1984, can be used when practicing the present invention.

Aerosol delivery systems of the type disclosed herein are available from numerous commercial sources including Fisons Corporation (Bedford, MA), Schering Corp. (Kenilworth, NJ) and American Pharmoseal Co. (Valencia, CA).

As will be understood by those skilled in the art, the exact dosage and frequency of administration of the bystander antigens of the present invention (in oral or aerosol form) is a function of the activity of the bystander antigen, as well as the age, sex, weight, and physical condition of the subject to be treated, and the concurrent administration or absence of other treatments. Consequently, adjustment of the dosages used and administration schedules must be determined based on these factors, and may need to be determined experimentally. Such determination, however, requires no more than routine experimentation, given the guidelines contained herein.

Experimental

In the examples below, which are intended to illustrate the present invention without limiting its scope, the following is demonstrated:

Example 1 is directed to the treatment of autoimmune diseases by oral administration of autoantigens. This series of experiments show that the active form of TGF-β1 isotype mediates suppression of $CD4^+$ T-cells specific to MBP and that $CD8^+$ T-cells induced by feeding MBP to animals cause the release of TGF-β and that it is TGF-β that is responsible for suppression. The same example also demonstrates that antigens that are not even specific to the tissues or organs under attack can elicit formation of T-suppressor cells which cause the release of TGF-β. This is illustrated by the oral administration of ovalbumin. The problem with ovalbumin, however, is that since it is not specific to the afflicted tissue, it is by itself incapable of targeting the T-suppressor cells to a site where cells contributing to attack can be found. (This problem is addressed in Example 2.) Example 1 also illustrates that not every orally administered antigen causes bystander-type suppression: bovine serum albumin does not.

Finally, Example 1 also demonstrates that the same mechanism (bystander suppression) is at work in suppression of experimental autoimmune encephalomyelitis (EAE) by oral administration of MBP. EAE has been studied in mice and other mammalian species as a model for multiple sclerosis (MS).

Example 2 is also related to the treatment of autoimmune diseases. This series of experiments show that an antigen capable of bystander suppression will upon oral administration cause the release of TGF-β and that, furthermore, if the suppressor T-cells elicited by this antigen can be recruited to a location where cells contributing to an attack can be found, those disease-promoting cells will be suppressed. Example 2 also shows that the suppressor T-cells that allow for the release of TGF-β do not have to encounter the suppressed cells in order for suppression to take place.

The way a non-specific bystander antigen can be rendered an efficient bystander (i.e. "forced" to cause TGF-β

to be released in the vicinity of the disease-promoting cells) is by substantially simultaneous injection with the same antigen (within 24 hours before or after bystander oral administration). For example, when OVA was fed to animals and then these animals were immunized with MBP/CFA to induce EAE, it was found that an injection with OVA would suppress EAE. This is due to the concentration of both EAE promoting cells (which the OVA-elicited suppressor T-cells do not recognize) and cells specific to OVA (which are specific to OVA, just as the OVA-elicited suppressor T-cells) in the lymph nodes of the animal. The implication of this showing for therapy is that non-specific bystander antigens could also be used in combatting retroviral-associated neurological disease if their suppressor T-cells can be targeted to a site where they would suppress disease-promoting cells.

Although both Examples 1 and 2 examine suppression of EAE, an autoimmune disease model system, these Examples provide data central to the present invention, even though the present invention does not directly involve autoimmune disease. First, these experiments establish that bystander antigen elicits suppressor T-cells which are specific to the antigen administered and this specificity results in localization of the cells to the antigen. Second, it is shown that although the T-cells are specific to the bystander antigen, the TGF-β from these T-cells suppresses the action of other immune cells in the area in a non-specific manner. It is these two aspects of bystander suppression which allow other diseases thought to be mediated by misdirected immune attack, such as retroviral-associated neurological disease, to be treated using the method outlined above. Specifically, such diseases can be treated by selecting the bystander antigen such that two events occur: 1) TGF-β is produced and 2) the suppressor T-cells producing the factor are localized, through proper selection of bystander antigen, to the tissue under cytotoxic attack.

Example 3 describes the production of recombinant human MBP and its purification. The protein is produced in a highly pure form, with no detectable proteolytically-derived fragments, in contrast to preparations of purified MBP from brain tissue. This rMBP protein (or fragments thereof) are useful as bystander antigen in the treatment of retroviral-associated neurological disease using the methods of the present invention. This is based on the ability of rMBP to elicit suppressor T-cells which produce TGF-β (as demonstrated in Examples 1 and 2), and the ability of this antigen, as a component of neural tissue, to target the elicited T-cells to the tissue under cytotoxic attack.

Example 4 shows that the human MBP recombinantly produced as described in Example 3 causes the in vitro proliferation of a human T-cell clone previously shown to react specifically with MBP. The data from this highly specific test of biologic activity indicates that recombinant production does not diminish the ability of MBP to act as a bystander antigen and has substantially the same activity as MBP extracted and purified from brain tissue.

Example 5 identifies the role of activated T-cells in HAM/TSP as shown by the ability of the infected T-cells to spontaneously proliferate in vitro and the ability of this proliferation to be elicited in non-infected cells through direct cell contact. This induction of proliferation in non-infected T-cells occurs even when the infected clones are fixed with formaldehyde or irradiated. Such large numbers of activated T-cells are thought to be significant in the development of retroviral-associated neurologic disease, as activated T-cells but not resting T-cells can cross the blood-brain barrier to initiate an inflammatory response in the CNS. This inflammatory response is precisely what is inhibited by the suppressor T-cells elicited by oral administration of bystander antigens, pursuant to the present invention.

EXAMPLE 1: Suppressor T-Cells Generated By Oral Tolerization To Myelin Basic Protein Suppress Both *In Vitro* And *In Vivo* Immune Responses By The Release Of TGF-β Following Antigen Specific Triggering

In the experiments described below the following materials and methods were used.

Animals. Female Lewis rats 6-8 weeks of age were obtained from Harlan-Sprague Dawley Inc. (Indianapolis, IN). Animals were maintained on standard laboratory chow and water ad libitum.

Antigens. Guinea pig myelin basic protein (MBP) was purified from brain tissue by the modified method of Deibler et al. (Prep.Biochem. 2:139,1972) as disclosed in U.S. Patent Application Serial No. 07/487,732 filed March 2, 1990. Protein content and purity were checked by gel electrophoresis and amino acid analysis.

Reagents. Commercial reagents used were as follows: monoclonal mouse anti-rat IFNγ neutralizing antibody (Amgen Biologicals, Thousand Oaks, CA); monoclonal hamster anti-murine TNFα+β antibody (Genzyme, Boston, MA); polyclonal rabbit anti-TGF-β_{1+2} neutralizing antibody (R & D Systems, Inc., Minneapolis, MN), and indomethacin (Sigma, St. Louis, MO). Turkey antiserum specific for the type 1 isoform of TGF-β was prepared as previously described (Danielpour, D., et al. J. Cell. Physiol. 138: 79-86,1989).

Induction Of Oral Tolerance. Rats were fed 1 mg of MBP dissolved in 1 ml PBS, or PBS alone, by gastric intubation using a 18-gauge stainless steel animal feeding needle (Thomas Scientific, Swedesboro, NJ). Animals were fed five times at intervals of 2-3 days with the last feeding two days before immunization. The purpose of this was to induce tolerance.

In Vitro Suppression Of Proliferative Responses By Supernatants. Spleen cells were removed 7-14 days after the last feeding and a single cell suspension prepared by pressing the spleens through a stainless steel mesh. For preparation of

supernatants, spleen cells at a concentration of 5 x 10^6 cells/ml were stimulated in vitro with MBP (50 μg/ml) in 10 ml of proliferation medium. Proliferation medium consisted of RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 2 x 10^{-5} M 2-mercaptoethanol, 1% sodium pyruvate, 1% penicillin and streptomycin, 1% glutamine, 1% HEPES buffer, 1% nonessential amino acids and 1% autologous serum. Supernatants were harvested at 24 hours and 100 μl added to 2.5 x 10^4 MBP specific T-cells, raised and maintained as previously described (Ben-Nun, A. et al., Eur. J. Immunol. 11:195-199, 1981), cultured with 5 x 10^5 irradiated (2500 rad) thymocytes, in 100 μl of proliferation media. MBP (50 μl/ml) was added to the culture in a volume of 20 μl. Experiments were performed in triplicate in round bottomed 96-well plates (Costar, Cambridge, MA). Cells were cultured for 72 hours at 37oC in an incubator with humidified 6% CO_2 and 94% air atmosphere, and each well was pulsed with 1 μCi of ^3H thymidine for the last 18 hours of culture. Cultures were harvested onto fiberglass filters using a multiharvester and counted using standard liquid scintillation techniques.

The purpose of this was to set up an assay system for soluble factors produced in oral tolerization.

Purification Of T-Cell Subsets. Depletion of lymphocyte subsets was performed by negative selection using magnetic beads according to a modified method of Cruikshank (J.Immunol. 138: 3817-3823,1987). Spleen cells were incubated with a 1:10 dilution of mouse anti-rat CD8, CD4, or B-cell monoclonal antibodies (mAbs) (clones OX/8, W3/25 or OX/33 respectively, commercially available from Serotec/Bioproducts, Indianapolis, IN) for 30 minutes on ice, washed twice, and then added to prewashed magnetic particles, with an average diameter of 4.5 μm (M-450) with goat anti-mouse IgG covalently attached (Dynal Inc., Fort Lee, NJ). The quantity of magnetic beads used was calculated as being 10 times the estimated target cell population. The cells were incubated with the beads in 0.5 ml

of RPMI 1640 supplemented with 10% fetal calf serum (FCS) in a 10 ml round bottomed test tube (Nunc) for 30 minutes on ice with gentle shaking every 5 minutes. After incubation, the bead/cell suspension was washed with 5 ml of medium and the cell-mAb-bead complexes were separated from unlabelled cells in a strong magnetic field using a magnetic-particle concentrator (Dynal-MPC-1) for two minutes. The supernatant was removed, and the procedure repeated twice to obtain the nonadherent fraction. The cells in the T-cell and B-cell depleted populations were >95% $CD4^+CD8^-$, $CD4^-CD8^+$ or $CD4^+CD8^+$ or $CD4^+CD8^+OX/33^-$ (B-cell depleted) as demonstrated by indirect flow cytometry. Whole spleen populations (5×10^6 cells) from MBP fed or control fed animals were cultured in the presence of MBP (50 μg/ml) in 1 ml of serum-free proliferation media. Depleted populations were cultured at a concentration of 2.5×10^6 cells/ml. Supernatants were collected at 24 hours and 100 μl added to responder cells as described above.

The purpose of this was to isolate specific subsets of T-cells in order to determine which T-cells were involved in Bystander Suppression.

Treatment Of Supernatants With Anti-Cytokine Antibodies. Spleen cells (5×10^6/ml in proliferation media) from MBP-fed and control animals were incubated in the presence of MBP (50μg/ml) plus neutralizing antibodies against interferon-gamma (INFγ), TGF-β, Tumor Necrosis Factor (TNF)$\alpha+\beta$ or with indomethacin for 72 hours. Antibodies were tested in a range of concentrations (1:250, 1:500, 1:1000) and indomethacin tested at concentrations of 0.5-1 μg/ml. At 24 hours, supernatants were collected and free antibody or antibody-cytokine complexes were removed using magnetizable polymer beads (Dynabeads, Dynal, Inc., Fort Lee, NJ). Beads coupled with anti-immunoglobulin antibodies were incubated at a concentration of 4×10^7 beads/ml for 30 minutes (done twice for each sample) and removed according to a modified method of

Liabakk et al. (Scand. J. Immunol. 30:641, 1989), using a Dynal Magnetic Particle Concentrator (Dynal, MPC-1).

The purpose of these experiments was to examine the soluble cytokines produced upon oral tolerization.

Measurement Of TGF-β Activity In Serum-Free Culture Supernatants. Serum free culture supernatants were collected as previously described (Kehri, et al. J. Exp.Med.163: 1037-1050, 1986; Wahl, et al. J.Immunol.145: 2514-2419,1990). Briefly, modulator cells were first cultured for 8 hours with MBP (50 μl/ml) in proliferation medium. Thereafter cells were washed three times and resuspended in serum-free medium for the remainder of the 72 hour culture, collected, then frozen until assayed. Determination of TGF-β content and isoform type in supernatants was performed using a mink lung epithelial cell line (American Type Culture Collection, Bethesda, MD #CCL-64) according to Danielpour et al. (supra), and confirmed by a Sandwich Enzyme Linked Immunosorbent Assay (SELISA) assay as previously described (Danielpour et al. Growth Factors 2: 61-71,1989). The percent active TGF-β was determined by assay without prior acid activation of the samples.

The purpose of these experiments was to measure and determine the isoform of the TGF-β produced by T-cells obtained from orally tolerized animals.

Immunization Of Animals. To induce a substantial EAE disease state, Lewis rats were immunized with 25 μg of MBP in 50 μl in the left food pad, emulsified in an equal volume of complete Freund's adjuvant containing 4 mg/ml of Mycobacterium tuberculosis (Difco).

In Vivo Administration Of Anti-TGF-β Antiserum And Control Sera. Turkey anti-TGF-β antiserum specific for the type 1 isoform was used for in vivo experiments and had previously been prepared and characterized (Danielpour et al., 1990, Supra). Serum was heat inactivated at 56oC for 30 min. before injection. Animals (5 per group) were injected intraperitoneally (I.P.) with anti-TGF-β antiserum or control

turkey serum at various concentrations (12.5, 25 or 50 μl diluted in PBS to a final volume of 100 μl), 5 times at days -2, 0, +2, +4, +6 in relationship to MBP/CFA immunization. 1 μl of the antiserum blocked 4 mg/ml of binding activity of ^{125}I-TGF-β1 to A549 cells (Danielpour et al., 1990, Supra). In vivo treatment was given both to orally tolerized animals and to animals to develop EAE without oral tolerization.

These experiments were performed to examine the effects of anti-TGF-β antiserum on oral tolerance induction in vivo, and to see whether TGF-β activity was abrogated.

Clinical Evaluation. To examine the correlation between in vitro assays and clinical disease, animals were evaluated in a blinded fashion every day for evidence of EAE. Clinical severity of EAE scored as follows: 0, no disease; 1, limp tail; 2, hind limb paralysis; 3, hind limb paraplegia, incontinence; 4, tetraplegia; 5, death. Duration of disease was measured by counting the total number of days from disease onset (usually days 10 or 11 after immunization) until complete recovery or death for each animal.

Delayed Type Hypersensitivity (DTH) Testing. DTH was tested by injecting 25 μg of MBP in PBS subcutaneously in the ear. Thickness was measured before and 48 hours after challenge, by a blinded observer, using micrometer calipers (Mitutoyo, Japan). Change in ear thickness pre-and post-challenge was recorded for each animal and the result expressed as the mean for each experimental group ± SEM.

DTH responses were monitored because they are mediated by CD4+ T-cells as is EAE.

Statistical Analysis. Comparisons of means were performed using a one-tailed student t-test and chi square analysis (as is known by those of ordinary skill in the art) was used in comparing the incidence of disease between groups.

Experiments were performed to determine whether supernatants collected from splenocytes depleted of T-cell subsets or B-cells from rats orally tolerized to MBP and

stimulated in vitro with MBP could suppress an MBP line. As shown in Figure 1, a reduction in the proliferation of the MBP line occurred with the addition of supernatants from B-cell depleted or CD4 depleted splenocytes from animals fed MBP and stimulated in vitro with MBP. No suppression occurred with supernatants from cells of Bovine Serum Albumin (BSA)-fed animals or CD8 depleted splenocytes from MBP-fed animals. This indicated that suppression was specific for the fed antigen and required suppressor T-cells.

In order to determine whether a known cytokine was responsible for mediating the suppression, neutralizing antibodies to cytokines postulated to have suppressor activity were added to the supernatants in an attempt to abrogate the suppression. As shown in Figure 2, rabbit anti-TGF-β antibody abrogated the suppression mediated by the supernatants in a dose-dependent fashion. No effect on suppression was seen with neutralizing antibodies to INFγ, TNFα+β, or when indomethacin, a prostaglandin blocker, was added. No suppression occurred when anti-TGF-β antibodies were added directly to the MBP specific responder T-cell line (data not shown). This indicates that TGF-β is responsible for the suppression observed in Fig. 1, and was due to a soluble factor.

In order to directly demonstrate the presence of TGF-β in supernatants of spleen cells from animals fed MBP and stimulated in vitro with MBP, supernatants were collected under serum-free conditions and assayed directly for TGF-β as described above. As shown in Figure 3, TGF-β was secreted by spleen cells from MBP fed animals stimulated in vitro in the presence, but not in the absence of MBP. Furthermore, TGF-β was also secreted when splenocytes from ovalbumin (OVA) fed animals were stimulated in vitro with OVA. Using a specific SELISA assay with blocking antibodies specific for either TGF-β1 or TGF-β2, it was further demonstrated that TGF-β was of the TGF-β1 isotype. In addition, the TGF-β secreted was in the active, rather than the latent form. The amount of TGF-β in

the group fed and stimulated in vitro with MBP was 6.8 ± 1.7 ng/ml with 68 ± 9% in the active form. In the OVA group the amount of TGF-β was 6.1 ± 1.0 ng/ml with 65 ± 9% in the active form. No active TGF-β was observed in supernatants from spleen cells of animals fed MBP and stimulated with a non-specific inducer of T-cell proliferation, concanavalin-A (Con-A), although small quantities (2.1 ± 0.45 ng/ml) of latent TGF-β were observed.

In order to determine whether TGF-β1 also played a role in suppression of EAE by oral tolerization to MBP, turkey anti-TGF-β1 anti-serum was administered in vivo. As shown in Figure 4A, paralytic EAE developed in control animals with a maximal disease severity between 3.2-3.5 on day 13 where the animals were injected with PBS or control turkey serum. Oral tolerization with MBP markedly reduced the severity of EAE (Figure 4C) in animals injected with PBS or control turkey serum. Maximal disease severity in animals treated 5 times with 50 μl of control serum was 3.2 ± 0.2 and in orally tolerized animals treated 5 times with 50 μl of control serum was 1.0 ± 0.2 ($p < 0.001$). As shown in Figure 4D, in vivo treatment with anti-TGF-β1 anti-serum abrogated protection induced by oral administration of MBP in a dose-dependent fashion; maximal disease severity in orally tolerized animals treated 5 times with 50 μl of anti-TGF-β1 anti-serum was 3.7 ± 0.2 vs. 1.0 ± 0.2 ($p < 0.001$, group D vs. C). Of note is that as shown in Figure 4B, there was a dose-dependent enhancement of disease in animals treated with anti-TGF-β1 anti-serum that were not orally tolerized to MBP. Disease onset was earlier, recovery was delayed, and disease severity was greater (4.5 ± 0.2 vs. 3.2 ± 0.2, groups B vs. A $p < 0.01$).

Delayed-type hypersensitivity (DTH) responses correlate with the clinical course of EAE and serve as a measure of in vivo cellular immunity to MBP (Brod, S. A. et al. Ann. Neurol. 29:615-622, 1991; Khoury, S. J. et al. Cell. Immunol. 131:302-310, 1990). DTH responses were tested in the

same groups described in Figure 4 by injecting 25 μg of MBP in PBS subcutaneously in the ear. Thickness was measured before and 48 hours after challenge. The change in ear thickness pre- and post-challenge was recorded for each animal and the results expressed as the mean for each experimental group ± SEM.

As shown in Figure 5 (A-D), prominent DTH responses developed in animals undergoing EAE and DTH responses were suppressed by oral administration of MBP. The suppressed DTH responses were abrogated by in vivo anti-TGF-β1 treatment in a dose-dependent fashion (1.5 ± 0.5 vs. 0.5 ± 0.3; $p < 0.001$, in animals injected 5 times with 50 μl of anti-TGF-β vs. control serum). Furthermore, following the same in vivo treatment, there was enhancement of DTH responses to MBP in animals recovering from EAE that were orally tolerized (2.1 ± 0.3 vs. 1.4 ± 0.3; $p < 0.01$ in animals injected 5 times with 50 μl anti-TGF-β vs. control serum).

The results presented above provide evidence for an immunoregulatory role played by endogenous TGF-β1 in the spontaneously occurring recovery from EAE and in the suppression of EAE induced by oral tolerization to MBP. In view of the fact that TGF-β features are highly conserved in evolution, it is anticipated that the immunosuppressive effects of TGF-β in experimental animals are similar to its effects in humans.

EXAMPLE 2: Antigen-Driven Bystander Suppression After Oral Administration of Antigens

In the experiments described below, the following materials and methods were used.

Animals. Female Lewis rats 6-8 weeks of age were obtained from Harlan-Sprague Dawley Inc. (Indianapolis, IN). Animals were maintained on standard laboratory chow and water ad libitum.

Antigens. Guinea pig MBP was purified from brain tissue by a method modified from Deibler et al. (supra) as described in Example 1 above and purity was checked by gel electrophoresis. Ovalbumin (OVA) and BSA were purchased from

Sigma Chemical Co. (St. Louis, MO) and keyhole limpet hemocyanin (KLH) from Calbiochem Behring Corp. (La Jolla, CA).

Immunization Of Animals. Animals were immunized with 25 μg of MBP in the footpad, emulsified in an equal volume of CFA containing 4 mg/ml of Mycobacterium tuberculosis (Difco Labs, Detroit, MI) in order to induce a substantial EAE disease state. For in vivo bystander suppression experiments, 50-300 μg of the secondary antigens OVA, BSA or KLH were injected subcutaneously in the same footpad in 100 μl PBS 8 hours after primary immunization with MBP CFA.

Clinical Evaluation. Animals were evaluated in a blinded fashion every day for evidence of EAE in order to correlate the clinical manifestations of Bystander Suppression with the in vitro assays described below. Clinical severity of EAE was scored as follows: 0, no disease; 1, limp tail; 2, hind limb paralysis; 3, hind limb paraplegia, incontinence; 4, tetraplegia; 5, death. Mean maximal clinical severity was calculated as previously described for each experimental group (7). Statistical analysis was performed using a one-tailed student's t test and a chi square analysis for comparing incidence between groups.

Induction Of Oral Tolerance. Animals were fed 1 mg MBP, OVA, BSA or KLH dissolved in 1 ml PBS or PBS alone, by gastric intubation using an 18-gauge stainless steel animal feeding needle (Thomas Scientific, Swedesboro, NJ). Animals were fed five times (total dose of 5 mg), at intervals of 2-3 days with the last feeding 2 days before immunization.

Delayed Type Hypersensitivity (DTH) Testing. DTH was tested by injecting 50 μg of MBP or OVA in PBS, subcutaneously into the ear. MBP was injected in the left ear and OVA in the right ear in the same animal. Thickness, in units of 0.01 inch, was measured in a blinded fashion, before and 48 hours after challenge, using micrometer calipers (Mitutoyo, Utsunomia, Japan). Change in ear thickness before and after challenge was recorded for each animal, and results were

expressed as the mean for each experimental group ± SEM; each group consisted of five animals.

Transwell Cultures. A dual chamber transwell culture system (Costar, Cambridge, MA), which is 24.5 mm in diameter and consists of two compartments separated by a semi-permeable polycarbonate membrane, with a pore size of 0.4 μm, was used. The two chambers are 1 mm apart, allowing cells to be coincubated in close proximity without direct cell-to-cell contact. To measure in vitro suppression of proliferative responses in transwell cultures, 5 x 10^4 MBP- or OVA-specific line cells, raised and maintained as previously described (Ben-Nun, A. et al., Eur. J. Immunol. 11:195, 1981), were cultured with 10^6 irradiated (2,500 rad) thymocytes, in 600 μl of proliferation media in the lower well. Spleen cells from orally tolerized rats or controls (fed BSA) were added to the upper well (5 x 10^5 cells in 200 μl). Spleen cells were removed 7-14 days after the last feeding, and a single cell suspension was prepared by pressing the spleens through a stainless steel mesh. MBP and OVA (50 μg/ml) were added in a volume of 20 μl. Because modulator cells are separated from responder cells by a semi-permeable membrane, they do not require irradiation. In some experiments, modulator cells were added in the lower well together with responder cells, and in these instances modulator cells were irradiated (1,250 rad) immediately before being placed in culture. Proliferation media consisted of RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 2 x 10^5 M 2-mercaptoethanol, 1% sodium pyruvate, 1% penicillin and streptomycin, 1% glutamine, 1 % HEPES buffer, 1% nonessential amino acids, and 1% autologous serum. Each transwell was performed in quadruplicate. The transwells were incubated at 37°C in a humidified 6% CO_2 and 94% air atmosphere for 72 hours. After 54 hours of culture, each lower well was pulsed with 4 μCi of [^{3}H]thymidine and at 72 hours split and reseeded to three wells in a round-bottomed 96-well plate (Costar) for harvesting onto fiberglass filters and counting using standard

liquid scintillation techniques. Percent suppression = 100 x (1 - Δ cpm responders cultured with modulators/Δ cpm of responders).

The transwell system was used to examine the soluble factors produced during Bystander Suppression and to monitor the transfer of suppression during the process.

Purification Of T-Cell Subsets. Depletion of T-cell subsets was performed by negative selection using magnetic beads according to the modified method of Cruikshank et al., supra. Spleen cells were incubated with a 1:100 dilution of mouse anti-rat CD8, or CD4, Mabs (clones OX/8 or W3/25 Serotec/Bioproducts, Indianapolis, IN) for 30 minutes on ice, washed twice, and then added to prewashed magnetic particles, with an average diameter of 450 microns (M-450) with goat anti-mouse IgG covalently attached (Dynal Inc., Fort Lee, NJ). The quantity of magnetic beads used was calculated as being 10 times the estimated target cell population. The cells were incubated with the beads in 0.5 ml of RPMI 1640 supplemented with 10% FCS in a 10 ml round-bottomed test tube (Nunc, Roskilde, Denmark) for 30 minutes on ice with gentle shaking every 5 minutes. After incubation, the bead/cell suspension was washed with 5 ml of medium and cell-mAB-bead complexes were separated from unlabeled cells in a strong magnetic field using a magnetic-particle concentrator (Dynal-MPC-1) for 2 minutes. The supernatant was removed, and the procedure repeated twice to obtain the nonadherent fraction. The T-cells in the depleted population were 95% $CD4^+CD8^-$ or $CD8^+CD4^-$ as demonstrated by indirect flow cytometry.

Adoptive Transfer Of Disease Suppression. In order to monitor the adoptive transfer of disease suppression occurring during Bystander Suppression donor rats were fed either 1 mg MBP, OVA, or KLH, five times at 2 day intervals and killed 7-14 days after the final feeding. Spleen cells were harvested, and incubated in vitro with the homologous antigen (50 μg/ml) in proliferation medium, for 72 hours. Cells were

injected intraperitoneally: 10^8 cells for whole spleen populations or 5-6 x 10^7 cells for CD8- or CD4-depleted populations. Recipient animals were irradiated (250 rad) before adoptive transfer, immunized with MBP/CFA 6 hours after adoptive transfer, and challenged 8 hours later with 50 μg OVA.

To determine whether cell-to-cell contact was required for in vitro suppression to occur, a transwell system (described above) was used. The results are set forth in Table 1 below.

As shown in Table 1, when irradiated splenocytes from MBP-fed animals were incubated together with an MBP line in the lower well, there was suppression of proliferation (line 2), while no suppression was observed with splenocytes from PBS fed animals (line 3). Virtually identical suppression was observed when modulator cells were separated from responder cells by the semipermeable membrane (lines 4 and 5). Thus, suppression appeared to be mediated by a soluble factor or factors that diffuse through the transwell membrane. Therefore, Bystander Suppression appeared to be operative in the induction of oral tolerance in EAE.

Table 1. Suppression of an MBP T-cell Line by Spleen Cells from MBP-fed Donors in Transwell System

	Upper well	Lower well	Δ cpm	Percent Suppression
1.	-	MBP line	37,809 ± 3,326	
2.	-	MBP line + MBP-fed modulators	18,412 ± 1,867	51
3.	-	MBP line + PBS-fed modulators	34,631 ± 3,994	8
4.	MBP-fed modulators	MBP line	15,620 ± 2,294	59
5.	PBS-fed modulators	MBP line	34,043 ± 3,731	10

5×10^4 MBP line cells + MBP (50 μg/ml) were placed in the lower well with 10^6 irradiated (2,500 rad) thymocytes as antigen presenting cells (APC). Splenic modulator cells (5×10^5) from MBP- or PBS-fed animals were added to either the upper or lower well. Modulator cells added to the lower well were irradiated (1,250 rad). Background counts of the MBP line without MBP added were between 1,000 and 2,000 cpm.

To determine whether that in vitro suppression observed in the transwell system required identical antigen specificity between modulator and responder cells, an OVA line was placed in the lower well. The results are set forth in Table 2 below.

As shown in Table 2, modulator cells from MBP-fed animals placed in the upper well were able to suppress an OVA line in the lower well, in the presence, but not in the absence, of MBP (lines 2 and 3). MBP added to modulator cells from animals fed PBS did not suppress the OVA line (line 4). Conversely, suppression of an MBP line was seen with modulator cells from OVA-fed animals in the presence of OVA (line 7). Of note is that soluble antigen added to the transwell in either well diffused across the membrane and thus was present in both wells as would be the case in vivo.

Table 2. Suppression of an OVA or MBP T-Cell Line by Spleen Cells from MBP- or OVA-f d Donors in Transwell System

	Modulator (upper well)	Responder (lower well)	Δ cpm	Percent Suppression
1.	-	OVA line + OVA	62,761 ± 3,881	-
2.	MBP-fed	OVA line + OVA	65,868 ± 3,989	-5
3.	MBP-fed + MBP	OVA line + OVA	30,974 ± 3,450	51
4.	PBS-fed + MBP	OVA line + OVA	61,132 ± 2,967	<1
5.	-	MBP line + MBP	71,503 ± 4,581	-
6.	OVA-fed	MBP line + MBP	67,075 ± 2,904	6
7.	OVA-fed + OVA	MBP line + MBP	37,778 ± 3,780	47
8.	PBS-fed + OVA	MBP line + MBP	68,104 ± 4,832	5

5×10^4 MBP or OVA line cells were placed in the lower well with 10^6 irradiated (2,500 rad) thymocyte as APC. Modulator cells (5×10^5) from MBP-, OVA- or PBS-fed animals were added to the upper well. Background counts of the MBP and OVA lines without MBP or OVA added were between 1,000 and 2,000 cpm.

To determine the relationship between the above *in vitro* bystander suppression and the *in vivo* situation, a series of experiments were conducted in the EAE model. Rats were fed OVA (1 mg, five times over a 10 day period), then immunized with MBP/CFA in the footpad and given OVA 8 hours later in the same footpad. As shown in Figure 6A, injecting OVA in the footpad 8 hours after immunization with MBP/CFA had no effect on EAE as expected. Mean maximal clinical disease severity was 3.9 ± 0.2 for MBP/CFA immunized and 3.8 ± 0.1 with OVA given subcutaneously. However, in animals fed OVA before immunization with MBP/CFA after which OVA was given subcutaneously in the footpad, suppression of EAE occurred in an analogous fashion to feeding MBP (Figure 6B); disease severity in OVA fed plus OVA given subcutaneously was 0.9 ±

0.2, in MBP fed it was 1.1 ± 0.1, and in the OVA fed and KLH given subcutaneously (control group) 3.9 ± 0.1 ($p < 0.001$, OVA and MBP fed vs. control). Therefore, CD4+ T-cells induced by immunization with MBP/CFA were down regulated by TGF-β released by CD8+ T-cells induced by oral administration of a bystander antigen, in this case OVA. No suppression of EAE was observed in animals fed OVA in whom KLH was given after MBP/CFA plus OVA subcutaneously (Figure 6C), disease severity was 3.7 ± 0.1 and 3.8 ± 0.2, respectively. These experiments demonstrate an in vivo effect similar to that seen in vitro in the transwell system. Specifically, modulator cells generated by oral tolerization to one antigen can suppress cells of a different antigen specificity when the tolerizing antigen is present.

To determine whether a correlation existed in the in vivo bystander system and to determine the degree of sensitization that occurs in association with the bystander effect, DTH responses were measured. Suppressed DTH responses to MBP were observed both in animals fed MBP and those fed OVA that were subsequently immunized with the MBP/CFA plus OVA (Figure 7). Oral administration of other antigens, such as KLH or BSA, had no effect on DTH responses to MBP in these animals. Feeding OVA followed by the injection of OVA subcutaneously in association with MBP/CFA did not generate an immune response to OVA as measured by DTH.

To rule out the possibility that something unique to OVA was responsible for the in vivo bystander suppression observed, similar experiments were conducted in which BSA was fed and then given subcutaneously after MBP/CFA immunization. As shown in Figure 8, oral administration of BSA prior to immunization with MBP/CFA followed by BSA (the bystander antigen) given subcutaneously suppressed EAE in an analogous fashion as that seen with OVA. Of note is that suppression of EAE associated with BSA was observed only when the secondary antigen was given subcutaneously at a dose of 300 μg, whereas with OVA, suppression occurred at a dose of 50 μg.

As shown in Figure 9, spleen cells from MBP- or OVA-fed animals adoptively transferred protection into naive recipients, which were immunized with MBP/CFA and given OVA subcutaneously. Furthermore, adoptively transferred suppression was abrogated by depletion of $CD8^+$ (suppressor T-cells), but not by depletion of $CD4^+$ cells. No protection was observed with the adoptive transfer of spleen cells from KLH-fed animals to animals immunized with MBP/CFA plus OVA.

EXAMPLE 3: Recombinant Expression and Purification of MBP

A cDNA coding for human MBP (commercially obtained from ATCC, Rockville, MD) was used as a PCR target with engineered restriction ends, and the cDNA fragment, beginning with an ATG coding for methionine, was ligated into the prokaryotic expression vector pAED4, a derivative of pET-3a (Studier et al., Methods in Enzymology 185: 6089, 1990) to produce pHO-MBP.T7. *E. coli* host BL21(DE3)pLysS (Novagen, Madison, WI) was transformed and grown in 100 µg/ml ampicillin and 20 µg/ml chloramphenicol to $O.D._{600}$ = 0.5. Cultures were made 1.0 mM IPTG and an additional 100 µg/ml ampicillin was added. Cultures were grown for an additional 3 h, cooled to 4°C, harvested and washed once with ice-cold STE (1 X STE contains 150 mM NaCl, 50 mM Tris-HCl, and 10 mM EDTA, pH = 7.4). Cell pellets were resuspended in 5 ml/100 ml culture of STE plus 0.4% Triton-X 100 and 0.2% sodium deoxycholate. To complete lysis, cells were frozen at -20°C overnight, thawed on ice, and made 0.2 mM PMSF, 1 µg/ml leupeptin, 2 U/ml aprotinin, and 10 mM $MgCl_2$. DNAseI was added to 40 µg/ml and RNAse to 10 µg/ml. The solutions were left on ice for an additional 30 min. An insoluble fraction was prepared by centrifugation at 20,000 x g for 30 min. at 4°C. The resulting inclusion bodies were washed twice with ice-cold STE containing 0.5% Triton-X 100, and resuspended in 8 M urea, 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, and 50 mM Tris-HCl pH = 8.0.

To purify recombinant MBP, 1.0 mg equivalent of pre-equilibrated CM-52 cellulose (Whatmann, Hillsboro, IL) per 1 liter culture was added to the sample, and rocked overnight at 4°C. Cellulose-MBP was washed twice with 20 volumes 2 M urea/0.02M sodium glycinate/0.02 M NaCl pH = 11.0, twice with 20 vol. deionized, distilled water, twice with 20 vol. 5 mM HCl, and eluted with 20 vol. 100 mM HCl, as previously described for brain MBP (Deibler et al., Prep. Biochem 2:139-165, 1972). Eluates were concentrated using Centriprep-10 concentrators (Amicon, Beverly, MA) and quantified using the BCA protein assay (Pierce, Rockford, IL). Samples were made 1 X Laemmli sample buffer (Laemmli, Nature 222:680-685) and electrophoresed on 12.5% SDS-polyacrylamide gels. Duplicate gels were either stained with Coomassie blue or transferred to nitrocellulose (Towbin et al., Proc. Natl. Acad. Sci. USA 76:4350-4354, 1979) and incubated with a mouse monoclonal antibody (IgG_1) directed against amino acid residues 130-137 of MBP (Boeringer Mannheim, Indianapolis, IN). Following incubation with donkey anti-mouse IgG coupled to alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA), the blot was developed with a one-step BCIP/NBT solution (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Upon development it was evident that the recombinantly produced MBP lacked the contaminating proteolytic fragments which are present in MBP isolated from brain tissue.

EXAMPLE 4: Biological Activity of Recombinantly Produced MBP

To access biologic activity, the recombinant protein of Example 3 (r-MBP) was tested in an in vitro proliferation assay using a human T-cell clone previously shown to react specifically with MBP. T-cells (clone OB.1A12.8) were plated in triplicate with irradiated antigen presenting cells (APC) for 72 hours in 96-well microtiter plates and pulsed with 2 uCi [^{3}H]-thymidine for the last 18 hours. APC were prepared by pulsing autologous peripheral blood mononuclear cells (PBMC) or

T-cell lines with 0, 0.1, or 1.0 μM of MBP peptide (amino acids 84-102), brain MBP, or r-MBP. If APC were not used, peptide, brain MBP, or r-MBP was added to the cultures at concentrations indicated. (LaSalle et al., J. Immunol. 147:774-780, 1991) The results are set forth in Table 3, below.

Table 3. T-cell Proliferation Assay using T-cell Clone Immunoreactive with MBP

Ag	conc. (μM)	PMBC (CPM +/- SEM)	none (CPM +/- SEM)
none	0	76 +/- 18	48 +/- 10
MBP 84-102	0.1	19,060 +/- 4,511	55 +/- 12
	1	174,708 +/- 1,493	34,673 +/- 11,373
	10	271,178 +/- 22,298	95,706 +/- 15,712
brain MBP	0.1	35,931 +/- 8,583	56 +/- 13
	1	89,795 +/- 19,185	8,112 +/- 2,602
	10	301,954 +/- 4,177	114,443 +/- 15,822
r-MBP	0.1	8,732 +/- 5,041	59 +/- 12
	1	221,477 +/- 37,537	1,594 +/- 429
	10	346,973 +/- 10,477	100,204 +/- 7,250

As seen in Table 3, T-cell clone Ob.1A12.8 responds to MBP peptide 84-102 presented by APCs in a specific and dose-dependent manner, as does MBP purified from brain. Pulsing the clones with rMBP resulted in equivalent stimulation of the cells, demonstrating first, that the cells will recognize and react to the protein, and second, that the recombinant protein is at least as effective in the proliferation assay as native protein. This result indicates that primary structure, and not higher-order structure or post-translational modifications such as acetylation or phosphorylation, is the principle determinant involved in T-cells recognition of MBP. Interestingly, when cells were incubated with the same antigen concentrations but in the absence of APCs, significant (30%) stimulation at higher doses of both MBPs is evident.

EXAMPLE 5: Role of Activated T-cells in HAM/TSP--T_{HTLV-I}-T-cell activation

Spontaneous T-Cell Proliferation of $CD4^+$ and $CD8^+$ HTLV-I-Infected T-Cell Clones. A series of T-cell clones were generated from the blood of two patients with HAM/TSP (patient DU, 48 clones; patient Pr, 45 clones). Seven of 40 T-cell clones from patient Du were positive for the HTLV-I *pol* region by PCR, indicating that these clones were infected with the virus. Southern blot analysis of genomic DNA performed on six clones (Du.4, Du.7, Du.20, Du.26, Du.34 and Du.43) confirmed the presence of HTLV-I-pro-virus and clonality. These T-cell clones represent *in vivo* infected T-cells, as allogeneic feeder cells were used for cloning and expansion. Five HTLV-I-infected clones were $CD4^+$ while one clone (Du.7) was $CD8^+$, demonstrating that both CD4 and CD8 populations can be infected by HTLV-I *in vivo* (Table 4). Eight of 24 clones from subject Pr were positive for HTLV-I tax mRNA by PCR as well as for HTLV-I antigens by Western blotting, both tests for viral infection (data not shown). Since these clones were generated by using autologous feeder cells, *in vitro* infection of T-cell clones cannot be excluded.

The growth characteristics of infected and noninfected T-cell clones were investigated. All clones required restimulation with mitogen and feeder cells at 10- to 14-day intervals for continuous growth. However, only HTLV-I-infected T-cell clones were found to proliferate in the absence of mitogen or interleukin-2 (IL-2) when cultured 7-10 days after the last stimulation, a phenomenon termed "spontaneous clonal proliferation" (Table 4). Thus, these T-cells exhibited growth characteristics of activated but not transformed cells. Of note is that clone Du.43 was positive for the HTLV-I *pol* region by PCR but did not proliferate spontaneously (Table 4). Southern blot analysis of genomic DNA confirmed the presence of HTLV-I proviral genome. However, no viral mRNA could be detected in this clone by Northern blotting.

HTLV-I-Infected T-Cells Induce Proliferation of Resting T-cells. As large numbers of activated T-cells are found in the blood of patients with HAM/TSP (Itoyama et al., Neurology 38:1302, 1988; Jacobson et al., Ann. Neurol. 23(supp.):S196, 1988), whether HTLV-I-infected T-cells could induce autologous T-cells to proliferate was examined. Irradiated or fixed HTLV-I-infected T-cell clones were found to induce proliferation of autologous blood T-cells or blood T-cells from allogenic normal subjects, while noninfected clones did not (Tables 5 and 6). Control T-cell clones infected with HTLV-I but not isolated from HAM/TSP patients, C91/PL and HUT-102, showed mild or no induction ability, respectively. (Table 5) The ability of fixed HTLV-I-infected T-cell clones to induce proliferation of resting blood T-cells indicated that T-cell surface structures and not soluble factors are important in the triggering of proliferation. Further, as it has been shown that T-cell activation is necessary for the cells to cross the blood brain barrier, this activation is significant in analyzing the function of T-cells in the inflammatory response seen in the CNS with patients afflicted with HAM/TSP.

Table 4. Spontaneous Proliferati n of $CD4^+$ and $CD8^+$ HTLV-I-infected T-cell Clones

Clone	Phenotype	HTLV-I infection	Proliferation, cpm
Du.3	ND	+	16,907
Du.4	CD4	+	31,305
Du.7	CD8	+	46,636
Du.20	CD4	+	2,209
Du.26	CD4	+	18,733
Du.34	CD4	+	5,495
Du.43	CD4	+	353*
Du.2	CD4	-	52
Du.5	CD4	-	187
Du.16	CD4	-	801
Du.19	CD4	-	317
DU.48	CD4	-	70

T-cell clones from HAM/TSP patient Du were established by single-cell cloning with phytohemagglutinin (PHA) and IL-2. Clones were tested by PCR amplification of the *pol* region and Southern blotting for the presence of the HTLV-I proviral genome. Spontaneous proliferation was determined by [^{3}H]thymidine incorporation.

* T-cell clone Du.43, which did not show spontaneous proliferation, was found to have integrated the HTLV-I proviral genome (genomic Southern blot) but had no viral RNA (Northern blot). ND, not determined.

Table 5. T-cell Activation by Fixed and Irradiated Clones

	[^{3}H]Thymidine incorporation, cpm		
	T_{Du}^+ clone Du.26	T_{normal}^+ clone Du.36	T_{normal}^+ clone Du.31
T-cells alone	20,428	108	108
Clones alone	10,962	10,962	130
Fixed clones	77	77	62
Fixed clones + T-cells	42,201	10,305	90
Irradiated clones	59	59	95
Irradiated clones + T-cells	90,965	116,480	452

Table 6. HTLV-I-infected T-cell Clones Induce Proliferation of Autologous T-cells

		[^{3}H] Thymidine incorporation, cpm				
			Irradiated stimulator T-cells			
T-Stimulator T-cells	HTLV-I infection*	Stimulator T-cells alone	Alone	With autologous T-cells†	With allogeneic T- cells‡	With autologous cells and autologous serum§
Pr. T-cells	+	584¶	29	503¶	597	---
Ctrl T-cells	-	43	26	64	34	---
Pr.G clone	+	10,702	30	19,749	31,437	---
Pr.17 clone	+	4,133	38	7,335	15,823	---
Pr.12 clone	-	83	24	60	109	---
Pr.15 clone	-	306	145	183	868	---
Pr.19 clone	-	36	18	63	29	---
C91/PL	+	3,023	805	3,051	4,014	---
Du. T-cells	+	173¶	151	173¶	---	163
Du.4 clone	+	18,891	25	43,507	---	60,229
Du.26 clone	+	21,474	46	63,977	---	80,076
Du.5 clone	-	2,148	36	2,344	---	4,180
Du.6 clone	-	985	36	2,309	---	3,721
Du.12 clone	-	137	41	1,863	---	4,167
Du.14 clone	-	ND†	ND†	753	---	3,079
HUT-102	+	1,732	1,639	1,639	---	1,648

* Stimulator T-cells were characterized by PCR and Southern blotting for the presence of tax mRNA (patient Pr) or *pol* retroviral sequences (patient Du).
† Stimulator T-cells were irradiated (5000 rad) and cocultured with T-cells from a HAM/TSP patient (Pr or Du).
‡ Stimulator T-cells were cocultured with resting T-cells from a normal subject.
§ Stimulator T-cells were irradiated (5000 rad) and cocultured with Du T-cells and 10% Du serum.
¶ Frozen T-cells from HAM/TSP patients were used, therefore T-cells did not proliferate to the same extent as fresh T-cells.

```
                                    10                                  20
Gly-Leu-Leu-Glu-Cys-Cys-Ala-Arg-Cys-Leu-Val-Gly-Ala-Pro-Phe-Ala-Ser-Leu-Val-Ala-

                                    30                                  40
Thr-Gly-Leu-Cys-Phe-Phe-Gly-Val-Ala-Leu-Phe-Cys-Gly-Cys-Gly-His-Glu-Ala-Leu-Thr-
               x

                                    50                                  60
Gly-Thr-Glu-Lys-Leu-Ile-Glu-Thr-Tyr-Phe-Ser-Lys-Asn-Tyr-Gln-Asp-Tyr-Glu-Tyr-Leu-

     S1
                                    70                                  80
Ile-Asn-Val-Ile-His-Ala-Phe-Gln-Tyr-Val-Ile-Tyr-Gly-Thr-Ala-Ser-Phe-Phe-Phe-Leu-

                                    90                                  100
Tyr-Gly-Ala-Leu-Leu-Leu-Ala-Tyr-Gly-Phe-Tyr-Thr-Thr-Gly-Ala-Val-Arg-Gln-Ile-Phe-

                                    100                                 120
Gly-Asp-Tyr-Lys-Thr-Thr-Ile-Cys-Gly-Lys-Gly-Leu-Ser-Ala-Thr-Val-Thr-Gly-Gly-Gln-

                                       S2
                                    130                                 140
Lys-Gly-Arg-Gly-Ser-Arg-Gly-Gln-His-Gln-Ala-His-Ser-Leu-Glu-Arg-Val-Cys-His-Cys-
```

Sequence of bovine PLP

```
Hum  A S Q K R P S Q R H G S K Y L A T A S T M D H A R H G F L P R H R D T
Bov  A A Q K R P S Q R - - S K Y L A S A S T M D H A R H G F L P R H R D T
Rab  A S Q K R P S Q R H G S K Y L A T A S T M D H A R H G F L P R H R D T
GPig A S Q K R P S Q R H G S K Y L A T A S T M D H A R H G F L P R H R D T
RatS A S Q K R P S Q R H G S K Y L A T A S T M D H A R H G F L P R H R D T
Chic A S Q K R S S F R H G S K - M A S A T S T D H A R H G S - P R H R D S

Hum  G I L D S I G R F F G G D R G A P K R G S G K D S H H P A R T A H Y G
Bov  G I L L D S S L G R F F G S D R G A P K R G S G K D S H H A A R T T H Y G
Rab  G I L L D S S I G R F F S S D R G A P K R G S G K D - - H A A R T T H Y G
GPig G I L L D S S I G R F F G S D R A A P K R G S G K D S H H A A R T T H Y G
RatS G I I L D D S I G R F F P S G D R G A P K R G S G K D S H - - T R T T H Y G
Chic G L L D S S L G R F F P T G D R - - V P R R G F G K D - - - A A R A S H V G

Hum  S L P Q K S - H G R T Q D E N P V V H F F K N I V T P R T P P P S Q G
Bov  S L P Q K A Q H G R P Q D E N P V V N F F K N I V T P R T P P P S Q G
Rab  S L P Q K S - H G R P Q D E N P V V N F F K N I V T P R T P P P S Q G
GPig S L F Q K S Q - - R S Q D E N P V V N F F K N I V T P R T P P P S Q G
RatS S L P Q K S Q - - - R T Q D E N P V V N F F K N I V T P R T P P P S Q G
Chic S I P Q R S Q H - R P(N,D,E,N)F V V N F F K N I V S P R T F F F M Q A

Hum  K G R G L S L S R F S W G A E G Q R P G F G Y G G R A S D Y K S A H K
Bov  K G R G L S L S R F S W G A E G Q K P G F G Y G G R A S D Y K S A H K
Rab  K G R G T V L S R F S W G A E C Q X P G F G Y G G R A - D Y K S - - K
GPig K G R G L S L S R F S W G A E C Q X P G F G Y G G R A - D Y K S - - K
RatS K G R G L S L S R F S W - - - - - - - - - - - - - - - - - - - - - -
Chic K G R G L S L T R F S W G G E G H K P G S G Y G G K F Y E H K S A H K

Hum  G F K G - V - D A Q G T L S K I F K L G G R - - - - D S R S G S P M A R R
Bov  G L K G - - H D A Q G T L S K I F K L G G R - - - - D S R S G S P M A R R
Rab  G L K G - A - D A Q G T L S R L F K L G G R - - - - D S R S G S P M A R R
GPig G F K G - A H D A Q G T L S K I F K L G G R - - - - D S R S G S P M A R R
RatS - - - - - - - - - - - - - - - - - - G G R - - - - D S R S G S P M A R R
Chic G H K G Y S H Q(G,E,G)T L S X I F K L G G R P(S,G,S,G,S)R S G S P V A R R
```

Amino Acids sequences of myelin basic proteins derived from human (Hum), bovine (Bov), rabbit (Rab), guinea pig (GPig), rat (RatS), and chicken (Chic) central nervous system tissue. The rate BP used was the 14 kDa BP (rat small, or RatS) which has a deletion of residues 118-159, coded for by the 6th exon of the BP gene. Both the human 18.5 and 17.2 kDa forms of BP were used. The latter has a deletion of residues 107-117 (underlined), coded for by the 5th exon of the BP gene. The sequences are arranged such that homologous residues from each species are arranged vertically, so that they can easily be compared with one another. This system accommodates the deletions and additions that are found among the species, and allows for a total of 177 potential sites among the different molecules. The sequences of residues in parentheses have not been established.

WHAT IS CLAIMED:

1. A method for treating retroviral-associated neurological disease in a mammal, the method comprising administering to said mammal an effective amount for treating said disease of a bystander antigen, said antigen eliciting the release of transforming growth factor beta (TGF-β) from suppressor T-cells wherein said TGF-β suppresses the T-cells contributing to the tissue damage characteristic of said disease.

2. The method of claim 1 wherein said bystander antigen is specific to neural tissue damaged during said disease.

3. The method of claim 1 wherein said bystander is administered to said mammal via oral route.

4. The method of claim 1 wherein said bystander is administered to said mammal via inhalation.

5. The method of claim 1 wherein:

said bystander antigen is administered by oral route or by inhalation;

said oral or inhalable bystander antigen elicits suppressor T-cells that cause the release of TGF-β;

said method further comprising also administering to said mammal the same bystander antigen via parenteral route, thereby causing said suppressor T-cells to be targeted to the same loci within the body of said mammal wherein the T-cells contributing to tissue damage are found.

6. The method of claim 1 wherein said disease is selected from the group consisting of HAM, TSP, and HIV-associated neurological disease and said bystander antigen is selected from the group consisting of myelin basic protein,

antigen, fragments thereof and combinations of any two of the foregoing.

7. The method of claim 1 further comprising administering to said mammal an amount of a synergist effective in combination with said bystander antigen to treat said disease.

8. The method of claim 1 wherein said bystander antigen is administered in a pharmaceutical oral dosage form, the form comprising:

an effective amount for treating said disease of a bystander antigen, said antigen eliciting the release of transforming growth factor beta (TGF-β) from suppressor T-cells wherein said TGF-β suppresses the T-cells contributing to the tissue damage characteristic of said disease; and

a pharmaceutically acceptable carrier or diluent.

9. The method of claim 8 wherein said bystander antigen is specific to neural tissue damaged during said disease.

10. The method of claim 8 wherein said disease is selected from the group consisting of HAM, TSP, and HIV-associated neurological disease and said bystander antigen is selected from the group consisting of myelin basic protein, proteolipid protein, a HTLV-I antigen, a HIV antigen, fragments thereof and combinations of any two of the foregoing.

11. The method of claim 8 further comprising administering to said mammal an amount of a synergist effective in combination with said bystander antigen to treat said disease.

12. The method of claim 1 wherein said bystander antigen is administered in a pharmaceutical inhalable dosage form, the form comprising:

an effective amount for treating said disease of a bystander antigen, said antigen eliciting the release of transforming growth factor beta (TGF-β) from suppressor T-cells wherein said TGF-β suppresses the T-cells contributing to the tissue damage characteristic of said disease; and

a pharmaceutically acceptable carrier or diluent.

13. The method of claim 12 wherein said bystander antigen is specific to neural tissue damaged during said disease.

14. The method of claim 12 wherein said disease is selected from the group consisting of HAM, TSP, and HIV-associated neurological disease and said bystander antigen is selected from the group consisting of myelin basic protein, proteolipid protein, fragments thereof, and combinations of any two of the foregoing.

15. The method of claim 12 further comprising administering to said mammal an amount of a synergist effective in combination with said bystander antigen to treat said disease.

FIG. 1

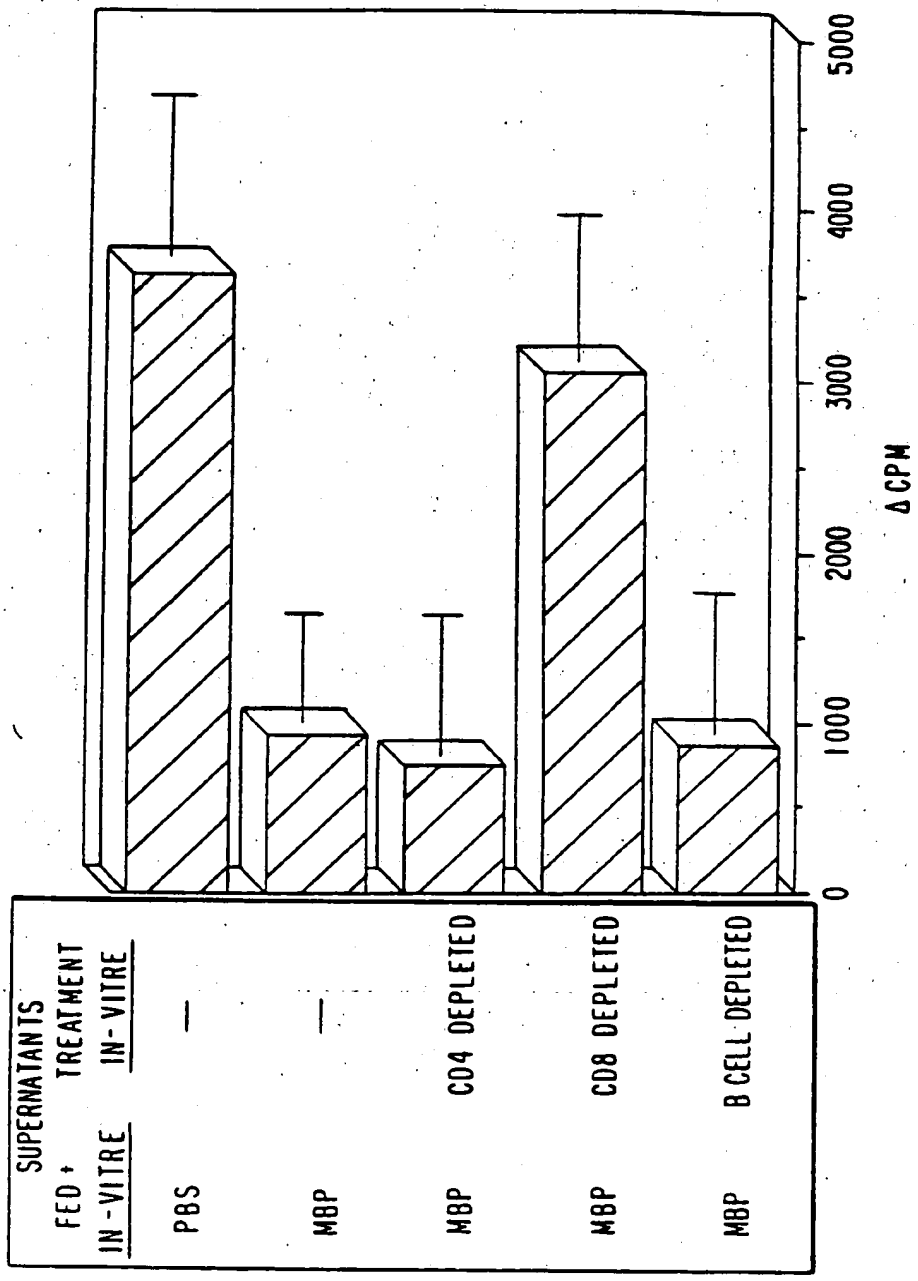

SUPERNATANTS
FED + TREATMENT
IN-VITRE IN-VITRE
PBS —
MBP —
MBP CD4 DEPLETED
MBP CD8 DEPLETED
MBP B CELL DEPLETED
0
1000
2000
3000
4000
5000
Δ CPM

FIG. 2

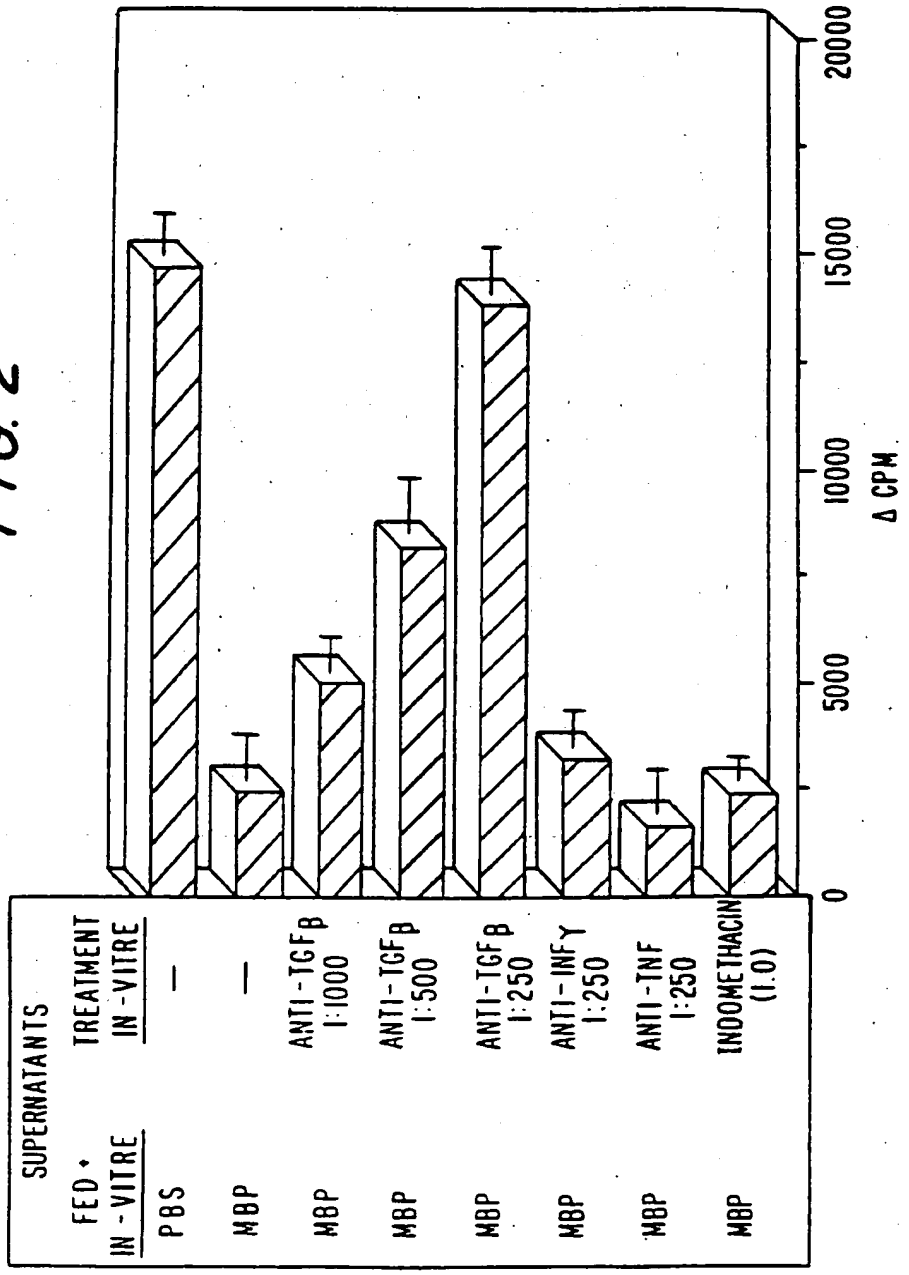

SUPERNATANTS
FED • IN-VITRE
TREATMENT IN-VITRE
PBS —
MBP —
MBP ANTI-TGFβ 1:1000
MBP ANTI-TGFβ 1:500
MBP ANTI-TGFβ 1:250
MBP ANTI-INFγ 1:250
MBP ANTI-TNF 1:250
MBP INDOMETHACIN (1.0)
0
5000
10000
15000
20000
Δ CPM

FIG. 3

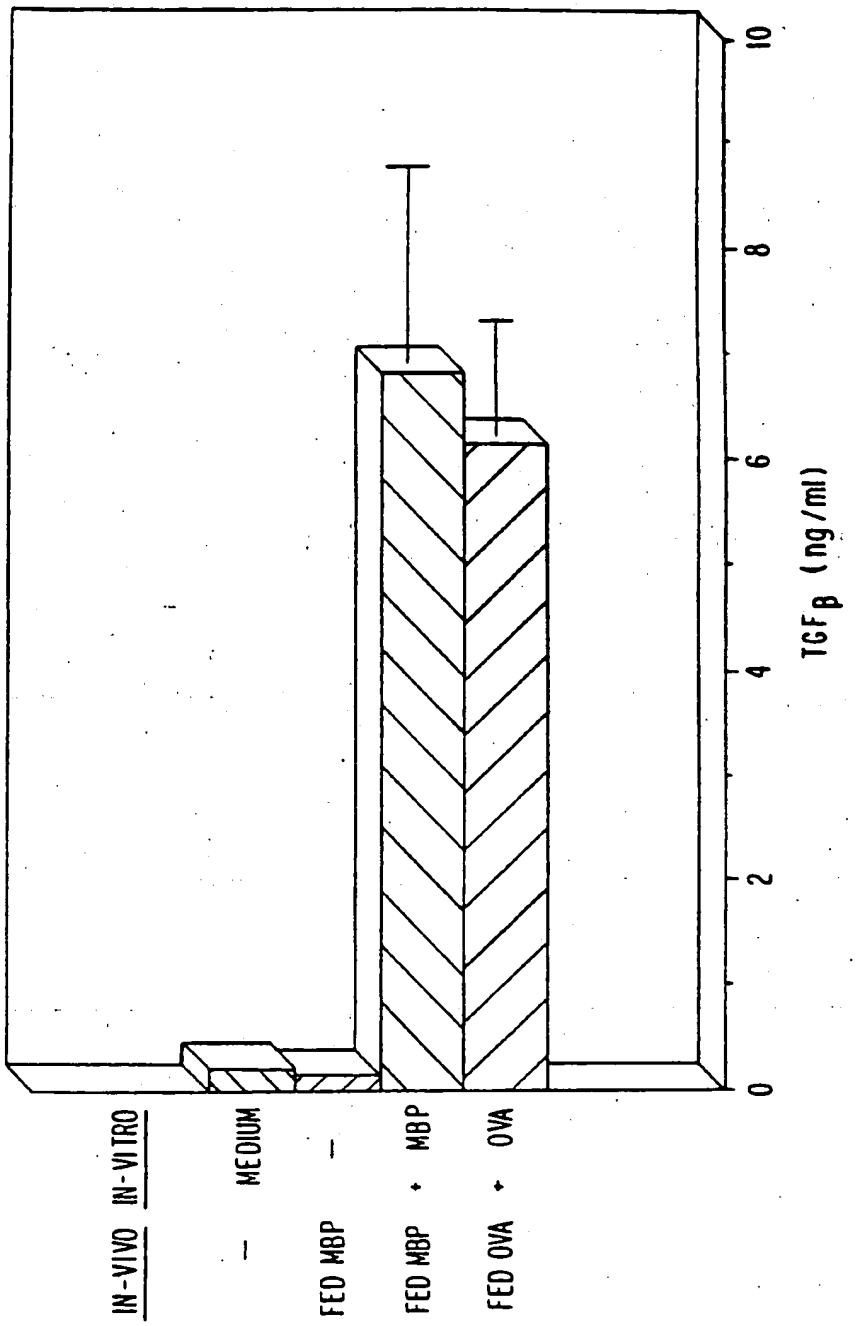

0
2
4
6
8
10
TGFβ (ng/ml)
IN-VIVO
IN-VITRO
—
MEDIUM
—
FED MBP
FED MBP + MBP
FED OVA + OVA

FIG. 4A

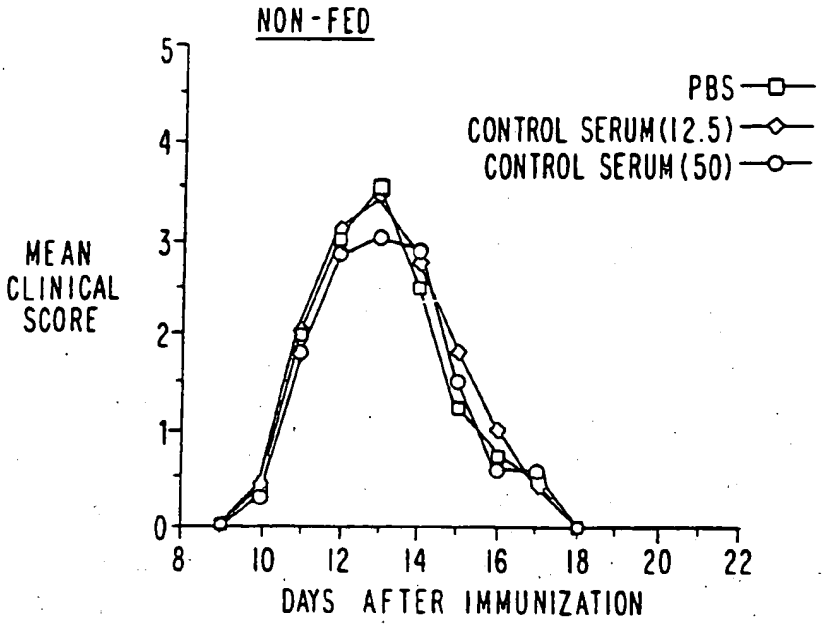

NON-FED
PBS
CONTROL SERUM(12.5)
CONTROL SERUM(50)
MEAN CLINICAL SCORE
0
1
2
3
4
5
8
10
12
14
16
18
20
22
DAYS AFTER IMMUNIZATION

FIG. 4B

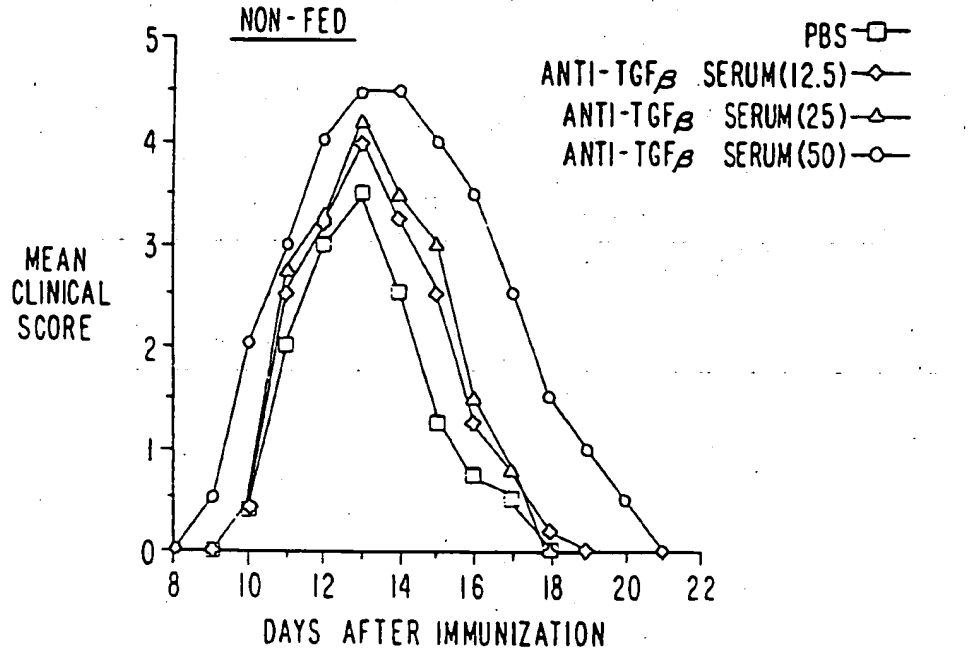

NON-FED
PBS
ANTI-TGFβ SERUM(12.5)
ANTI-TGFβ SERUM(25)
ANTI-TGFβ SERUM(50)
MEAN CLINICAL SCORE
0
1
2
3
4
5
8
10
12
14
16
18
20
22
DAYS AFTER IMMUNIZATION

FIG. 4C

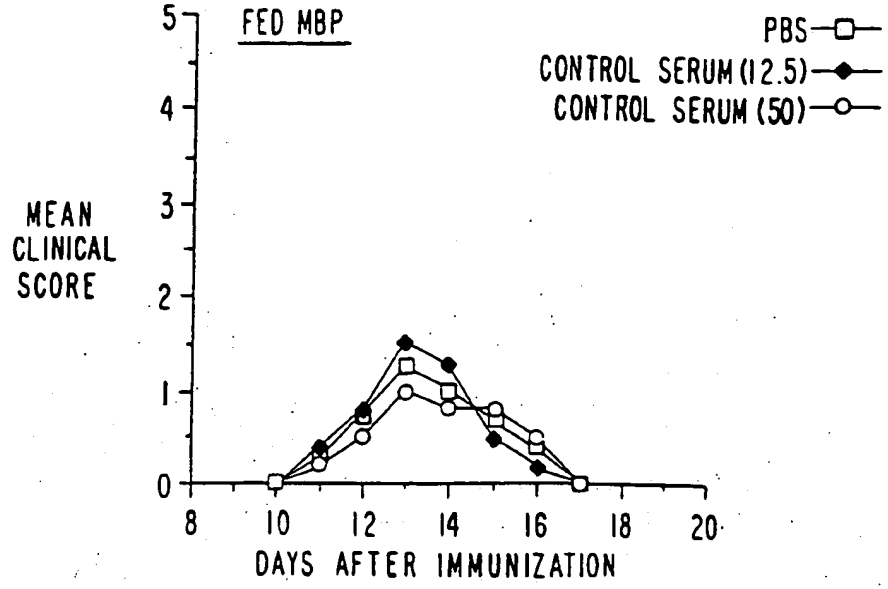
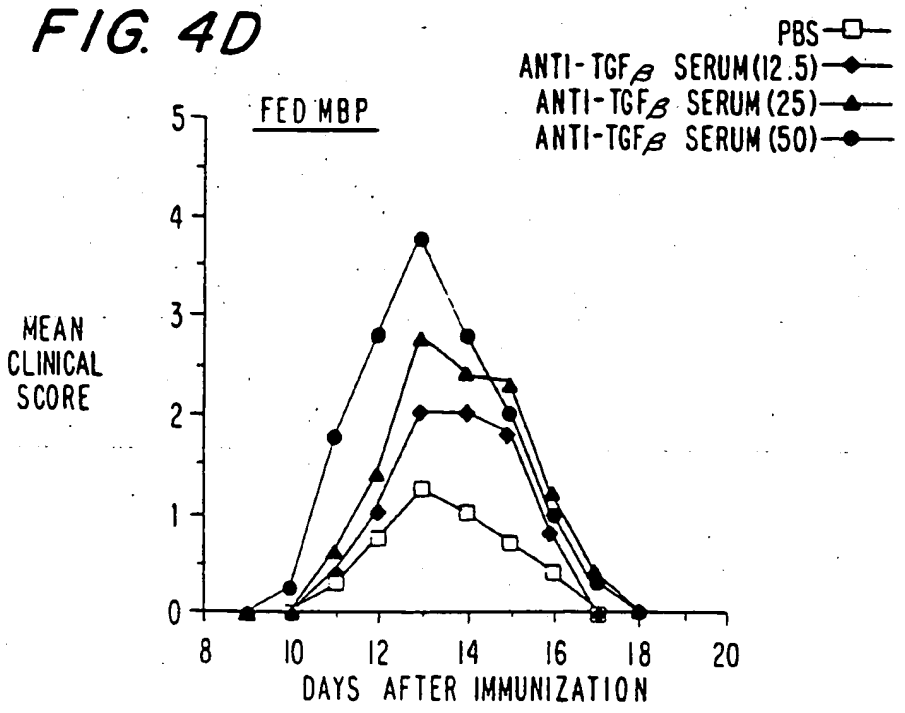

FED MBP
PBS
CONTROL SERUM (12.5)
CONTROL SERUM (50)
MEAN CLINICAL SCORE
0
1
2
3
4
5
8
10
12
14
16
18
20
DAYS AFTER IMMUNIZATION

FIG. 4D


FED MBP
PBS
ANTI-TGFβ SERUM (12.5)
ANTI-TGFβ SERUM (25)
ANTI-TGFβ SERUM (50)
MEAN CLINICAL SCORE
0
1
2
3
4
5
8
10
12
14
16
18
20
DAYS AFTER IMMUNIZATION

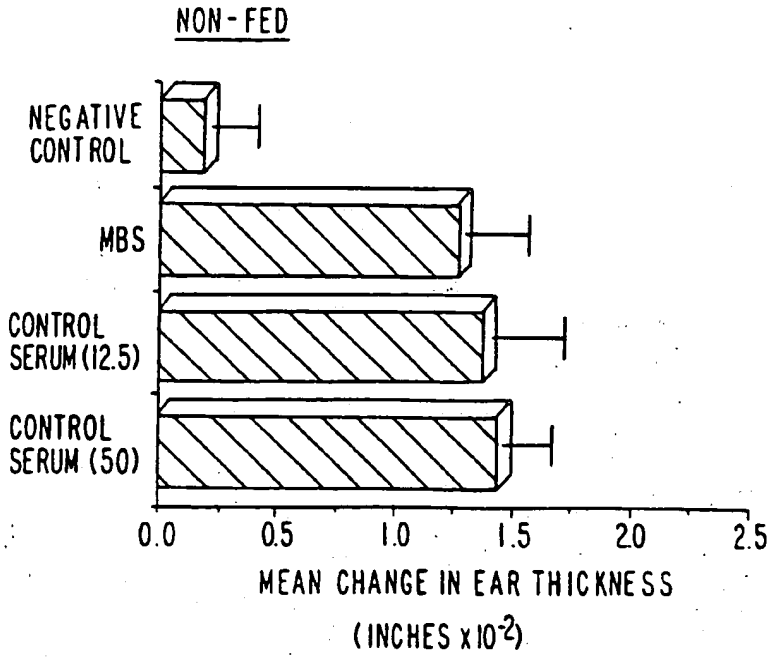

NON-FED
NEGATIVE CONTROL
MBS
CONTROL SERUM (12.5)
CONTROL SERUM (50)
0.0
0.5
1.0
1.5
2.0
2.5
MEAN CHANGE IN EAR THICKNESS
(INCHES x 10-2)

FIG. 5A

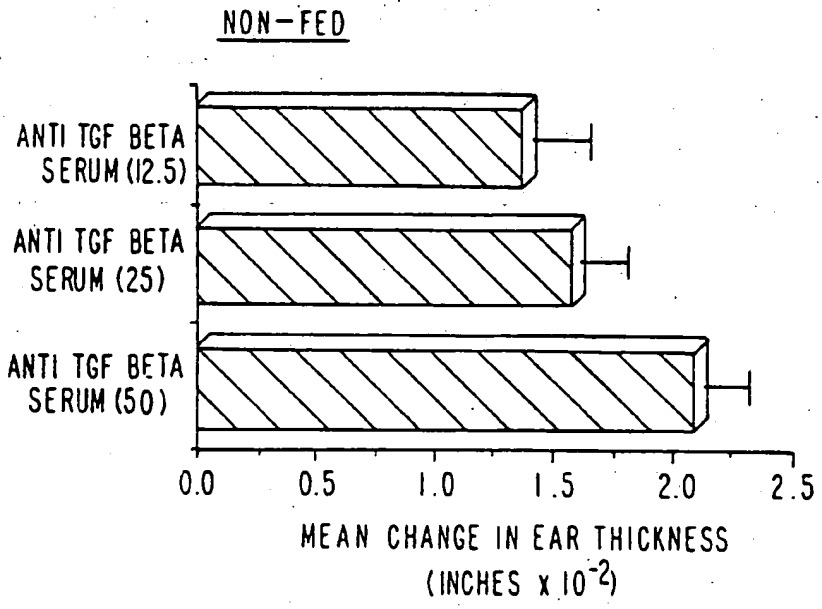

NON-FED
ANTI TGF BETA SERUM (12.5)
ANTI TGF BETA SERUM (25)
ANTI TGF BETA SERUM (50)
0.0
0.5
1.0
1.5
2.0
2.5
MEAN CHANGE IN EAR THICKNESS
(INCHES x 10-2)

FIG. 5B

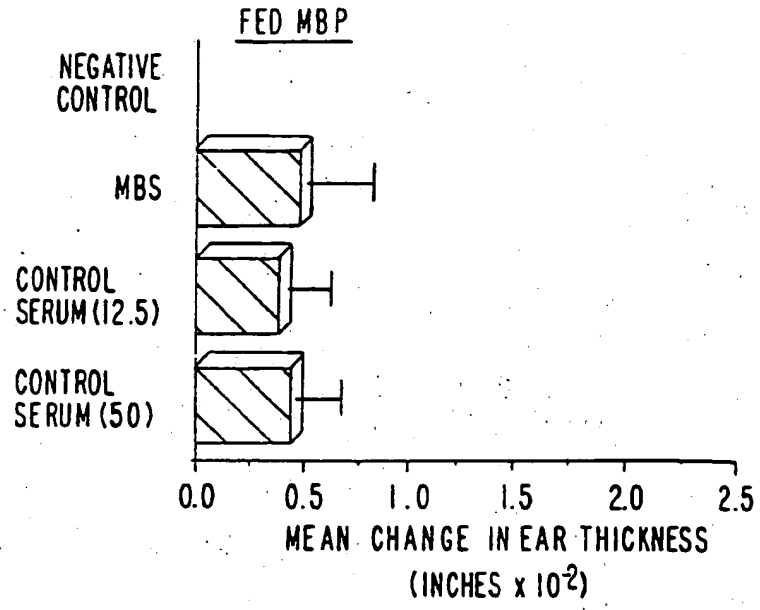

FED MBP
NEGATIVE CONTROL
MBS
CONTROL SERUM (12.5)
CONTROL SERUM (50)
0.0
0.5
1.0
1.5
2.0
2.5
MEAN CHANGE IN EAR THICKNESS
(INCHES x 10^{-2})

FIG. 5C

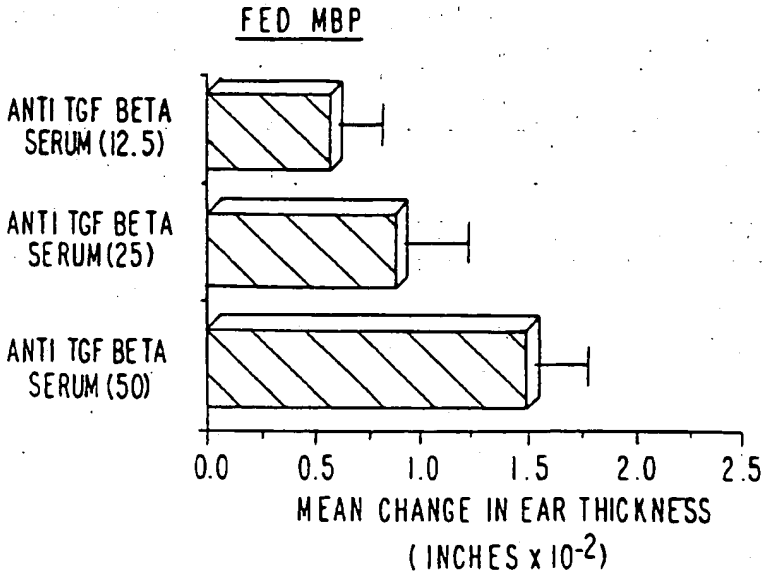

FED MBP
ANTI TGF BETA SERUM (12.5)
ANTI TGF BETA SERUM (25)
ANTI TGF BETA SERUM (50)
0.0
0.5
1.0
1.5
2.0
2.5
MEAN CHANGE IN EAR THICKNESS
(INCHES x 10^{-2})

FIG. 5D

FIG. 6A

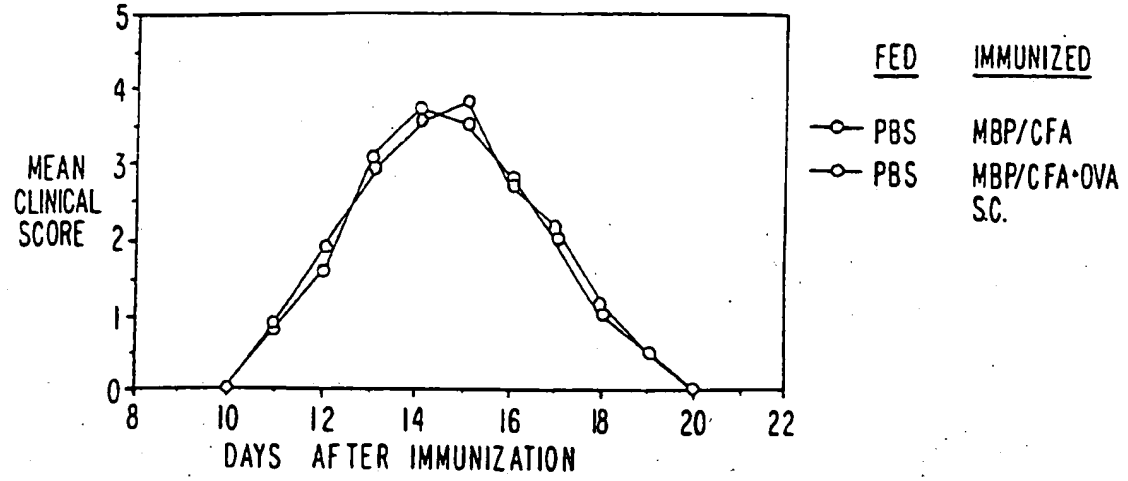


FIG. 6B

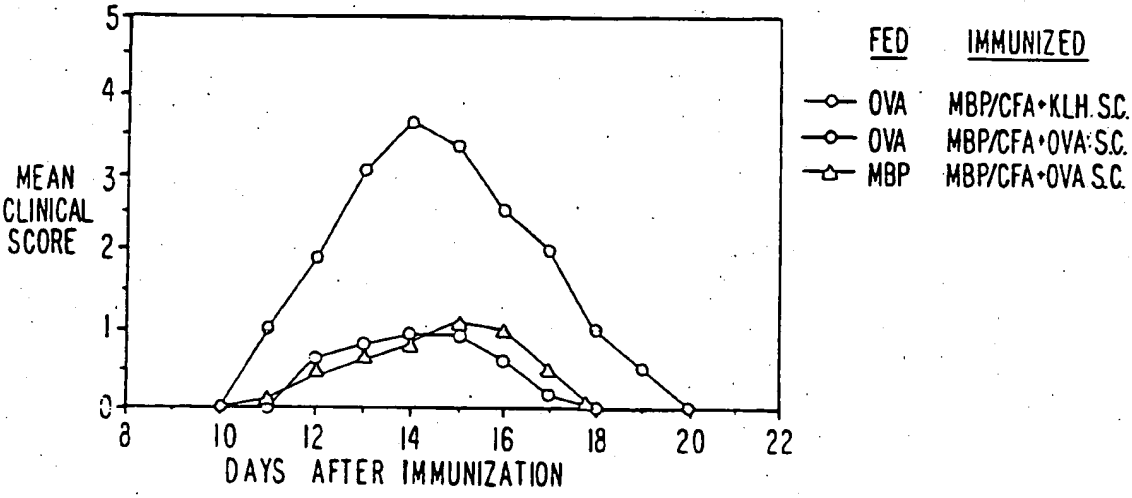
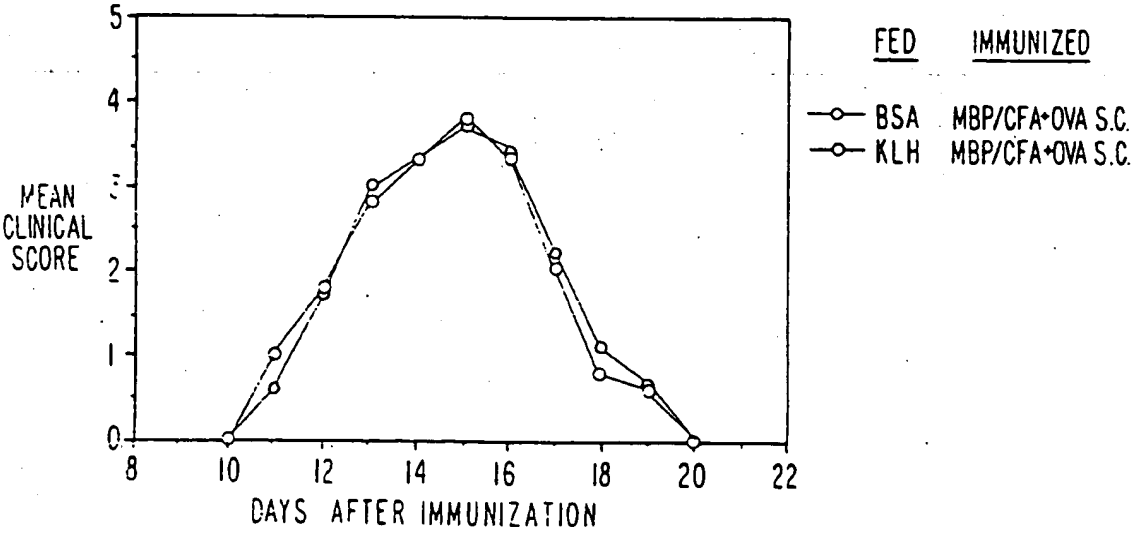


FIG. 6C



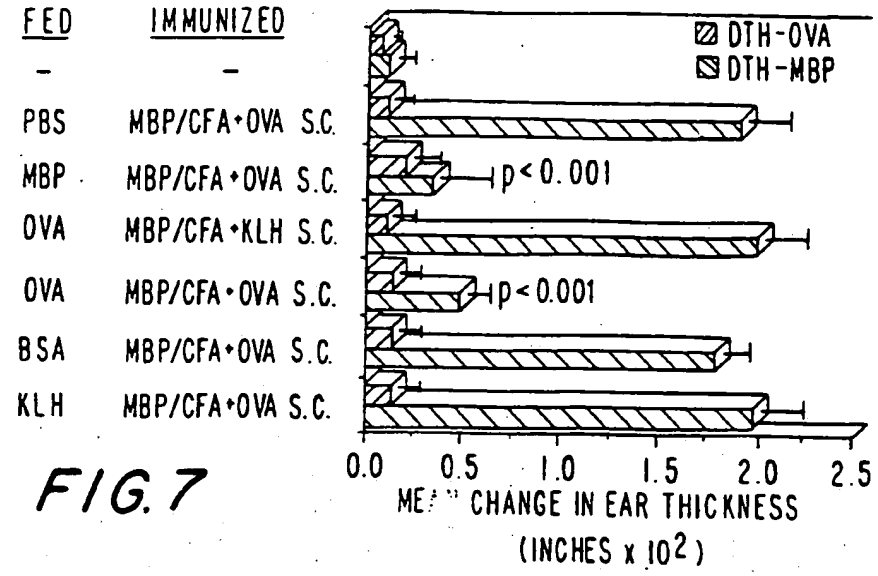


FIG. 7

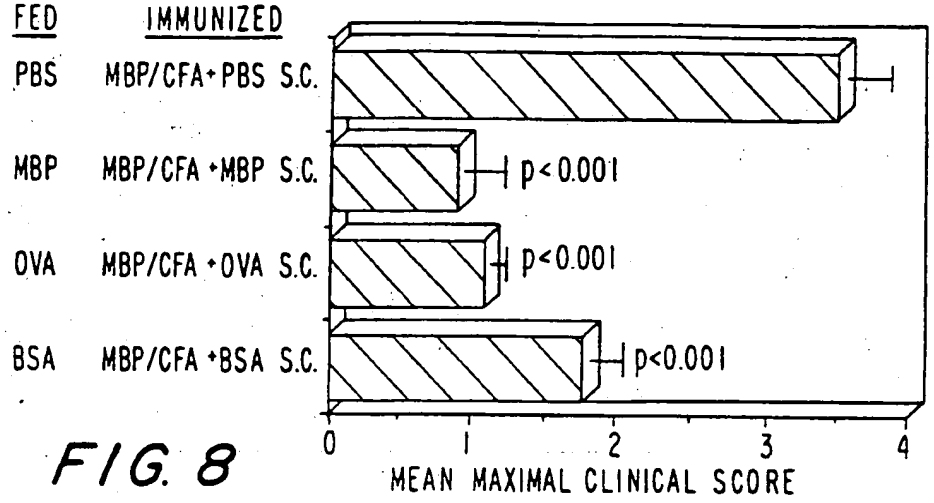


FIG. 8

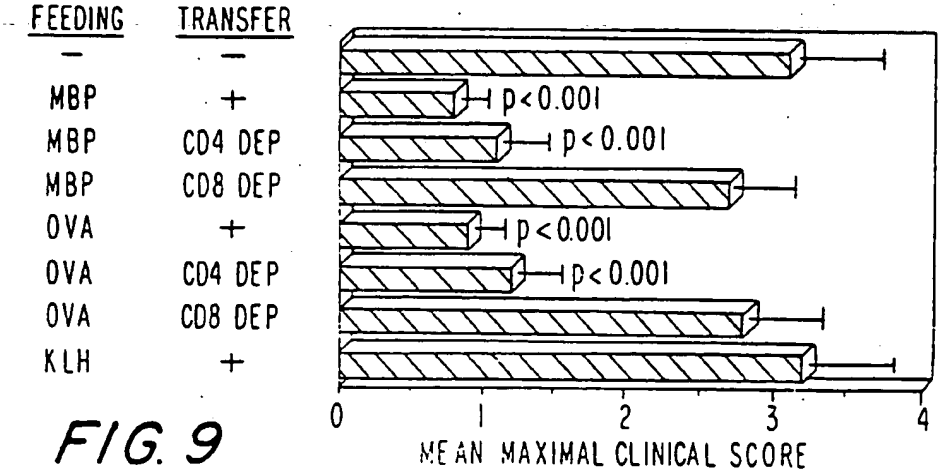


FIG. 9

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US93/07786

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/02, 39/21; C07K 13/00, 15/04
US CL :424/88; 530/351

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/88; 530/351, 826; 514/885, 903

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Medline, Chem Abstracts, Biosis, Embase, Scisearch, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	US, A, 5,190,750 (Otsuka et al) 02 March 1993, see column 3, line 59, column 5, lines 24 and 36, and column 6, line 54.	1-15
Y	Science, Vol. 252, issued 05 April 1991, J. Marx, "Testing of Autoimmune Therapy Begins", pages 27-28, see page 27.	1-15
Y	Journal of Immunology, Vol. 146, No. 4, issued 15 February 1991, W. J. Karpus et al, "$CD4^+$ Suppressor Cells Inhibit the Function of Effector Cells of Experimental Autoimmune Encephalomyelitis Through A Mechanism Involving Transforming Growth Factor-β", pages 1163-1168, see page 1165.	1-15

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 22 OCTOBER 1993	Date of mailing of the international search report 01 NOV 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer JULIE KRSEK-STAPLES Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/07786

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Clark et al (Newman), "Aerosols and the Lung" published 1984, see pages 197-224, especially page 197.	4, 12
Y	B.N. Fields et al (Hirsch), "Virology", published 1990 by Raven Press Ltd. (N.Y), see pages 1545-1570, especially page 1556.	6, 10, 14
Y	B.N. Fields et al (Cann), "Virology", published 1990 by Raven Press, Ltd. (N.Y.), see pages 1501-1527.	6, 10, 14